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TITLE: Development of Coactivator-Dependent, First-in-Class Therapies for Breast Cancer

PRINCIPAL INVESTIGATOR: Bert O'Malley

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX 77030

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O'Malley, Bert W

E-Mail:berto@bcm.edu

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One Baylor Plaza
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

By integrating multiple signaling pathways that cancer cells rely on for growth and survival, p160 steroid receptor coactivator (SRC) family members (SRC-1, SRC-2/TIF2/GRIP1, SRC-3/AIB1/pCIP/RAC3) represent emerging targets for anti-cancer drug development. During this reporting period, we have characterized and published our work on two potent and selective small molecule inhibitors (SMIs), bufalin and verrucarin A, against SRCs from high throughput screening efforts. Cryo-electron microscopic analyses of DNA/estrogen receptor/SRC-3 protein complexes achieved by our group are providing powerful new insights into understanding the conformation of intact, full length proteins in a complex and should provide valuable new information on the mechanism of action of SRC SMIs as well.

15. SUBJECT TERMS
Breast cancer, steroid receptor coactivator, estrogen receptor, cryo-electron microscopy, small molecule inhibitors

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TABLE OF CONTENTS

1. INTRODUCTION:

Transcription factors in mammals require coactivators to mediate their transcriptional activities. Through modulating gene expression regulated by hormones, growth factors, and cytokines, coactivators play crucial roles in many biological and pathological processes including cell proliferation, differentiation, carcinogenesis, and metastasis. The best studied include the p160 steroid receptor coactivator (SRC) family, which contains three members: SRC-1, SRC-2 (TIF2, GRIP1, or NCOA2), and SRC-3 (AIB1/ACTR/NCOA3). These coactivators have been implicated in a wide number of cancer types and deserve strong consideration as key molecular targets for a next generation of chemotherapeutics. SRC-3 expression is upregulated significantly in breast cancers and correlates with HER2 positivity, disease recurrence in HER2-positive BCs and resistance to endocrine therapy. We intend to translate SRC family small molecule inhibitors (SMIs) into effective therapeutic drugs for the treatment of human breast cancers. Here we describe our work on the execution a of high throughput (HTP) screen with secondary *in vitro* assays that will be followed by preclinical animal model testing. Breakthrough advances in Cryo-electron microscopic analyses of DNA/NR/SRC-3 protein complexes achieved by our group are providing powerful new insights into understanding the conformation of ERα in complex with DNA and coactivators and should provide valuable new information on the structural mechanism of action of SRC SMIs, as well as interacting drugs. We envision that SRC-3 SMIs will be first-in-class chemotherapeutic agents that, in combination with existing endocrine, targeted and chemotherapeutic agents, can be used to treat breast cancer patients.

2. KEYWORDS:

Breast cancer, steroid receptor coactivators, estrogen receptor, cryo-electron microscopy, small molecule inhibitors

3. ACCOMPLISHMENTS:

Major goals of the project:

Task 1. Perform SRC small molecule inhibitor (SMI) compound screening. (Months 1-18):
75% complete

Task 2. Structure activity relationship (SAR) assessment of SRC SMIs. (Months 12-48):
Initiating work on this task

Task 3. SRC-3 – p300 - ER – DNA holocomplex cryo electron microscopy (Cryo-EM). (Months 1-36): **50% complete**

Task 4. Preclinical testing of SRC SMIs. (Months 18-60):
Not started – will be initiated later this year

Task 5. Clinical testing of SRC SMIs. (Months 48-60):
Not started – will be initiated next year

Accomplishments under these goals (tasks):

SRC Small molecule inhibitor screening and characterization (Task 1): We completed high throughput screening for SRC-3 and SRC-1 SMIs using large compound libraries; compounds that can inhibit the transcriptional activity of SRCs were identified that are much more potent than our originally identified tool compounds. This HTP screen explored an extensive range of chemical space using a library of nearly 400,000 compounds. In addition to this, we have also completed high throughput screens for SRC-2 SMIs. These screens, which were done in collaboration with Scripps Institute in Florida, led to the identification of more than 500 compounds in total (http://pubchem.ncbi.nlm.nih.gov), (AID 588352, AID588354 and AID651957). Follow up analysis of these hits has led to the identification of the compound bufalin, as a potent SMI. Bufalin was able to promote downregulation of SRC-1, SRC-2 and SRC-3 at low nanomolar concentrations. This contrasts with our original tool compound, gossypol that is effective at mid-micromolar levels. Follow up work is being conducted to characterize the many new compounds that we have identified to have a panel of the most effective SRC-3 SMIs possible for further characterization.

We have focused on examining the effects of bufalin on cell motility and invasion in breast cancer cell lines. We have found that bufalin can inhibit cell motility in a cell culture-base cell motility "wound" assay. We also have found that bufalin can strongly suppress breast cancer cell metastases in an MDA-MB-231 mouse xenograft model and are planning to perform similar experiments testing its effects in blocking tumor progression in ER+ breast cancer cells as well. More details on our characterization of bufalin are contained in our Wang et al. manuscript (see **Appendix A**).

We also have expanded our characterization of bufalin and 20+ other SRC SMIs including verrucarin A and diacetoxyscirpenol (see below) to assess their ability to inhibit the growth of ER+, HER2+ and ER+/HER2+ breast cancer cell lines *in vitro*. Numerous potentially repositionable drugs are being characterized and most of these compounds are able to reduce the cellular protein concentration of SRCs and inhibit cancer cell growth. We also have tested the ability of these SRC-3/SRC-1 SMIs to block cancer cell growth when used in combination with other anti-cancer drugs. SRC-3/SRC-1 SMIs are particularly effective when combined with PI3K/AKT/mTOR pathway inhibitors such as BEZ235 and MK2206. In conclusion, we have made considerable progress in expanding our understanding of the cellular pathways that regulate SRC-3/SRC-1 activity and protein stability. We plan to leverage this information to drive our efforts to develop potent and selective SRC-3/SRC-1 SMIs that we plan to further develop into anti-cancer drugs for clinical use.

Medicinal chemistry (Task 1): Bufalin shows promising activities against breast cancer cell growth through the inhibition of SRCs. However, bufalin has toxicity to the heart, and medicinal chemistry modification is therefore needed to find non-toxic derivatives with improved anticancer activity. Bufalin is a natural product with multiple asymmetric centers, representing a great challenge for chemical synthesis. A comprehensive medicinal chemistry study of bufalin is

not feasible. Our testing of ~10 commercially available bufalin analogs, such as digoxin, digoxigenin, digitoxin, ouabain, cinobufagin, resibufogenin and strophanthidin, suggested that the steroid core of bufalin (except for the 14β-OH) seems to be critical to the activity. Our medicinal chemistry plan is therefore to modify the 17-, 14- and 3-positions. There have been numerous efforts for total synthesis of bufalin as well as related cardioglycoside toxins during the past few decades. Based on these literature methods, we spent several months in finding practical synthetic methods for the medicinal chemistry study. As shown in the scheme at right starting from dehydroandrosterone, a readily available compound, we have now established a synthetic route, through which the medicinal chemistry modifications at the 17-position of bufalin, may be achieved. Four bufalin analogs containing an ethynyl, 2-methoxypyridin-5-yl and phenyl substituent at the 17-position have been successfully synthesized (**Fig. 1**). Biological activity testing of these compounds is on-going in the laboratories of Drs. Lonard and Palzkill.

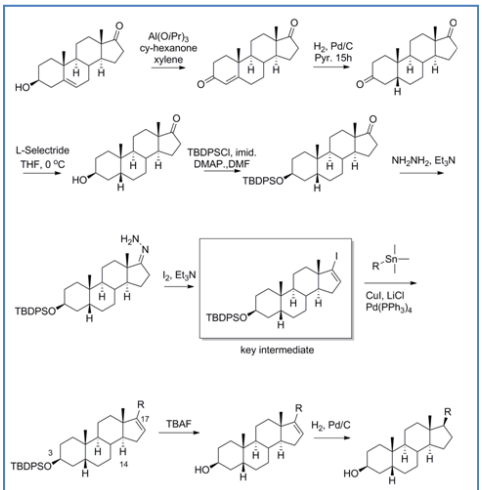

Fig. 1. Medicinal chemical modification of bufalin. Strategy used for the synthesis of bufalin derivatives using a common chemical precursor.

Going forward, the following medicinal chemistry goals will be:

- To synthesize ~50 bufalin analogs with a broad chemical diversity, in order to find compounds with improved inhibitory activity against SRCs as well as breast cancer.
- To establish a synthetic method to make bufalin analogs with a 14β-OH feature;
- To establish a synthetic method to modify the 3-position of bufalin;
- To analyze the structure activity relationship (SAR) between bufalin derivatives and , in an effort to rationally design compounds with improved activity.

SRC-3 SMI binding Studies (Task 1): Biochemical compound binding experiments have been performed to measure changes in the intrinsic fluorescence of domains of SRC-3 in the presence of lead chemicals. Fluorescence spectroscopy revealed that bufalin was able to bind the SRC-3 bHLH domain at low nanomolar concentrations.

We also have used fluorescence spectroscopy to establish possible direct interactions of SRC-3 with two additional promising chemicals: verrucarin A and diacetoxyscirpenol and to further localize the interaction sites on the SRC-3. Dose dependent measurements of the intrinsic fluorescence emission of three domains of SRC-3 were performed to assess the effects of these chemicals. These chemicals affect the intrinsic fluorescence of the SRC-3 domains both with respect to the emission intensity and the emission peak wavelength. Verrucarin A quenches the fluorescence of the bHLH, RID and CID domains of SRC-3 but shifts the emission peak to a shorter wavelength. The effects of diacetoxyscirpenol on these domains are either too limited or too variable to ascertain if there are interactions between SRC-3 domains and this chemical.

However, these chemicals appear to have small effects on the fluorescence of these domains at concentrations below 10 nM (this is the lowest concentration we tested here). We are planning to carry out more detailed investigations with fresh proteins to confirm the low concentration effect of these chemical on the fluorescence peaks of these SRC-3 domains and to determine their half-affecting concentrations with greater precision.

Verrucarin A as a SRC-3 selective SMI (Task 1): Verrucarin A was identified as a SMI that can selectively promote the degradation of the SRC-3 protein, while affecting SRC-1 and SRC-2 to a lesser extent and having no impact on CARM-1 and p300 protein levels (**Fig. 2**). Verrucarin A was cytotoxic toward multiple types of cancer cells at low nanomolar concentrations, but not toward normal liver cells. Importantly, verrucarin A was able to kill MCF-7 breast cancer cells at doses equivalent to those required to promote SRC-3 protein downregulation (**Fig. 3**). Moreover, verrucarin A was able to inhibit expression of the SRC-3 target genes MMP2 and MMP13 and attenuated cancer cell migration. We found that verrucarin A effectively sensitized cancer cells to treatment with other anti-cancer drugs. Binding studies revealed that verrucarin A does not bind directly to SRC-3, suggesting that it inhibits SRC-3 through its interaction with an upstream effector. In conclusion, unlike other SRC SMIs characterized by our laboratory that directly bind to SRCs, verrucarin A is a potent and selective SMI that blocks SRC-3 function through an indirect mechanism. More details on our characterization of bufalin are contained in our Yan et al. manuscript (see **Appendix A**).

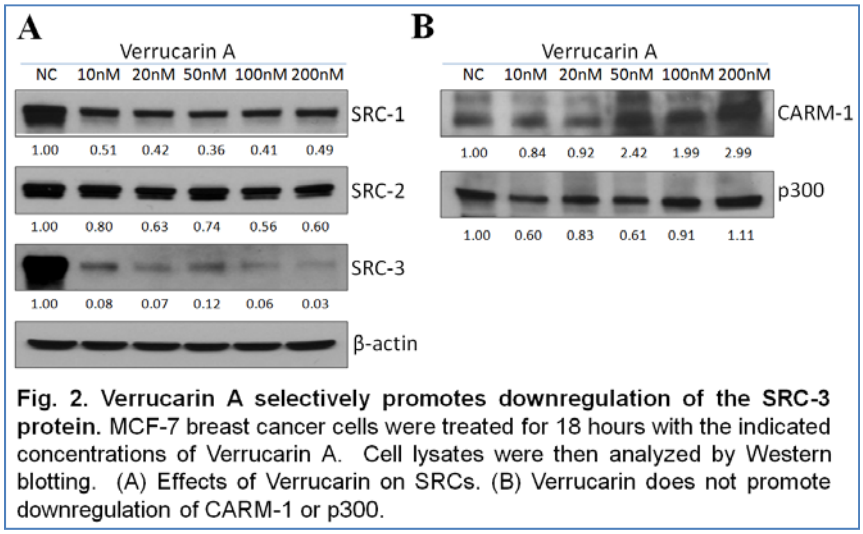

Fig. 2. Verrucarin A selectively promotes downregulation of the SRC-3 protein. MCF-7 breast cancer cells were treated for 18 hours with the indicated concentrations of Verrucarin A. Cell lysates were then analyzed by Western blotting. (A) Effects of Verrucarin on SRCs. (B) Verrucarin does not promote downregulation of CARM-1 or p300.

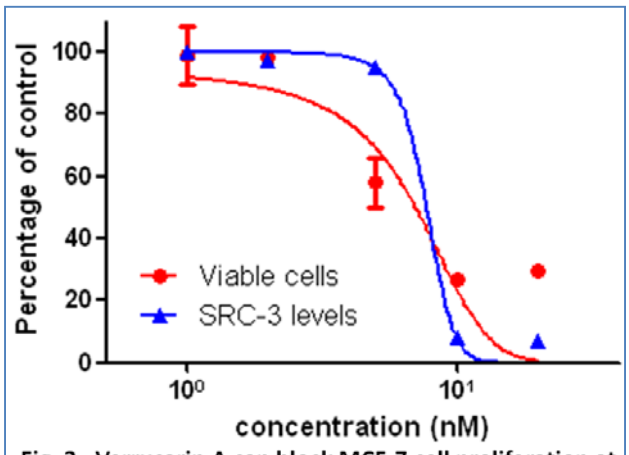

Fig. 3. Verrucarin A can block MCF-7 cell proliferation at concentrations equivalent to that which promotes SRC-3 protein downregulation. MCF-7 cells were treated with increasing concentrations of verrucarin A and cell viability was assessed via MTS assay (red line). The effects of verrucarin A were determined in parallel (blue line).

Biochemical compound binding experiments have been performed to measure changes in the intrinsic fluorescence of domains of SRC-3 in the presence of additional lead chemicals including emetine, cephaeline and diacetoxyscirpenol. Dose dependent measurements of the intrinsic fluorescence emission of three domains of SRC-3 were performed to assess the effects of these chemicals. These chemicals affect the intrinsic fluorescence of the SRC-3 domains both with respect to the emission intensity and the emission peak wavelength. The effects of diacetoxyscirpenol on these domains are either too limited or too variable to ascertain if there are interactions between SRC-3 domains and this chemical. However, these chemicals appear to have small effects on the fluorescence of these domains at concentrations below 10 nM (this is the lowest concentration we tested here). We are planning to carry out more detailed investigations to confirm the low concentration effect of these chemical on the fluorescence peaks of these SRC-3 domains and to determine their half-affecting concentrations with greater precision.

Characterization of a novel SRC Small Molecule Stimulator as an anti-cancer agent (Task 1): By integrating multiple signaling pathways that cancer cells rely on for growth and survival, p160 steroid receptor coactivator (SRC) family members (SRC-1, SRC-2/TIF2/GRIP1, SRC-3/AIB1/pCIP/RAC3) represent emerging targets for anti-cancer drug development as we have discussed before. A number of compounds have been characterized as potent and selective small molecule inhibitors (SMIs) against SRCs from high throughput screening efforts including

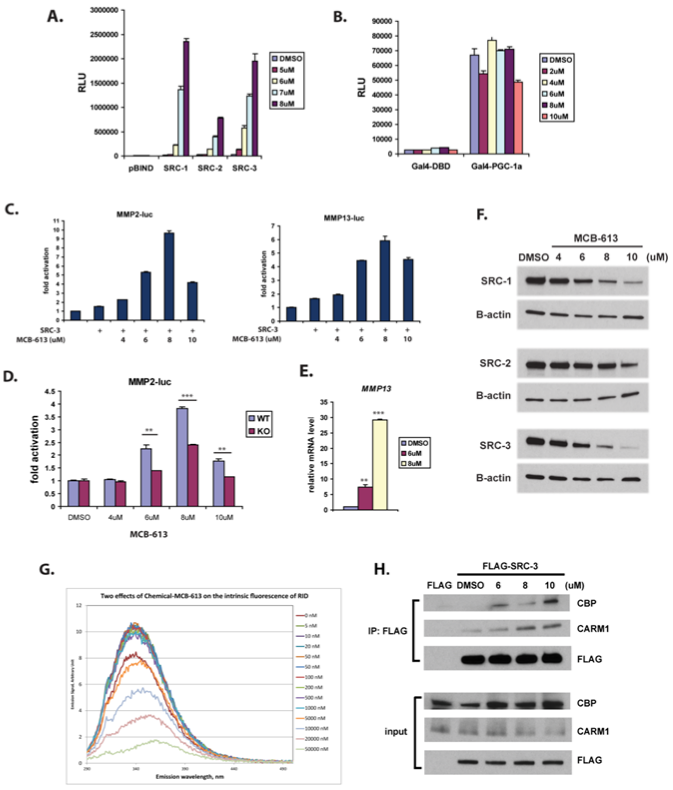

Fig. 4. MCB-613 hyper-stimulates SRC coactivator activity. (A) The intrinsic transcriptional activity of a GAL4 DNA binding domain fused SRC-1, SRC-2 and SRC-3 was assessed using a GAL4 responsive lucirerase reporter. Cells were treated with the indicated concentrations of MCB-613 for 18 hours. (B) The intrinsic transcriptional activity of PGC-1α was not affected by MCB-613 treatment. (C) MCB-613 treatment stimulates expression of the SRC-3 target gene MMP2-luc. (D) Deletion of the SRC-3 gene via a zinc-finger nuclease based technology (KO) leads to lower induction of MMP2 upon MCB-613 treatment. (E) The expression of another SRC-3 target gene, MMP13, is elevated by MCB-613 treatment. (F) Protein levels for all three SRCs are downregulated in MCB-613 treated cells. (G) MCB-613 binds directly to the receptor interacting domain of SRC-3 based on cell-free binding measurements. (H) MCB-613 treatment induces the rapid formation of a SRC-3/CARM1/CBP coactivator protein complex. Cells were treated with compound for 1 hour, followed by co-IP analyses.

bufalin and verrucarin A which we have recently published on. Unexpectedly, these screening efforts uncovered numerous compounds that are able to stimulate SRC activity as well. We are in the process of characterizing one of these compounds, MCB-613 as a novel small molecule stimulator (SMS) capable of strongly driving the transcriptional activity of all three SRCs (**Fig 4**). Genetic knockout or pharmacological inhibition of SRCs with existing SMIs can block the effects of MCB-613. Investigation of the underlying molecular mechanism responsible for MCB-613 mediated SRC stimulation revealed that it activates SRCs by increasing their interaction with other coactivators and induces the cellular unfolded protein response (UPR) which is coupled to the generation of reactive oxygen species (ROS) and the activation of the Abl kinase-SRC activation axis (**Fig. 5**). Importantly, we were able to exploit the SRC SMS effects of MCB-613 to induce excessive stress specifically in cancer cells, suggesting that over-stimulation of the SRC oncogenic program may be an effective strategy to kill cancer cells that are already utilizing cellular stress response pathways to their fullest extent.

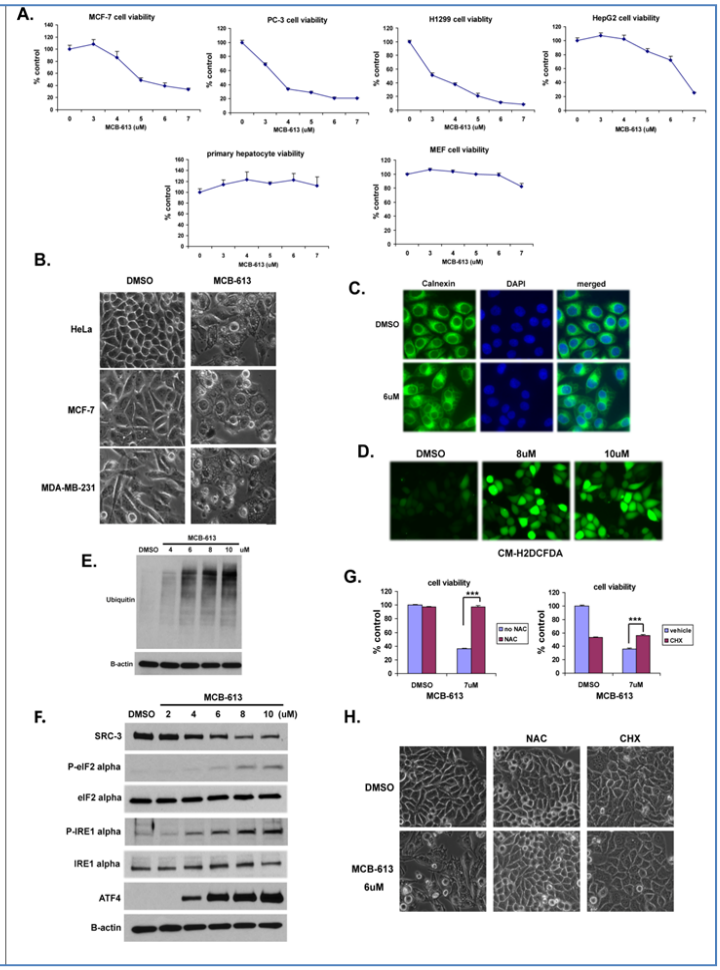

Figure 5. Compound MCB-613 induces ROS and cancer cell selective cytotoxicity. (A) MCB-613 selectively kills cancer cells. MCF-7, PC-3, H1299, HepG2, mouse primary hepatocytes and mouse embryonic fibroblasts were treated with different concentrations of MCB-613 for 48 hours. Cell viability was determined by MTS assay. (B) MCB-613 treatment causes extensive cytoplasmic vacuolization. HeLa, MCF-7 and MDA-MB-231 cells were treated with MCB-613 for 24 hours. (C) Vacuoles induced by MCB-613 are derived from endoplasmic reticulum. HeLa cells were treated with MCB-613 for 24 hours, followed by immunostaining with antibody against calnexin. (D) MCB-613 treatment causes rapid increase of intracellular ROS level. HeLa cells were pre-loaded with a general ROS indicator CM-H2DCFDA, followed by MCB-613 treatment for 30 minutes. (E) MCB-613 treatment leads to proteasome dysfunction. HeLa cells were treated with MCB-613 for 5 hours, followed by Western blot with antibody against ubiquitin. (F) MCB-613 treatment induces markers of unfolded protein response (UPR). HeLa cells were treated with MCB-613 for 4 hours followed by Western blot with antibodies against SRC-3, phosphorylated eIF2 alpha, total eIF2 alpha, phosphorylated IRE1 alpha, total IRE1 alpha, ATF4 and β-actin. (G) Cell death caused by MCB-613 can be rescued by treatment with an antioxidant or protein synthesis inhibitor. HeLa cells were treated with MCB-613 for 24 hours in the presence or absence of N-Acetyl cysteine (NAC) or cycloheximide (CHX). (H) Cytoplasmic vacuolization caused by MCB-613 was blocked by antioxidant and protein synthesis inhibitor treatment.

Cryo-electron microscopic analysis of the ERa – coactivator – DNA complex (Task 3):

No prior structural information exists on the receptor-coactivator complex to complement pre-existing and sometimes controversial biochemical information. During this reporting period, we used cryo-EM to determine the quaternary structure of an active complex of DNA-bound ERα,

steroid receptor coactivator 3 (SRC-3) and a secondary coactivator (p300). Identification of the protein components in this complex is aided by cryo-EM maps of p300 monoclonal antibodies

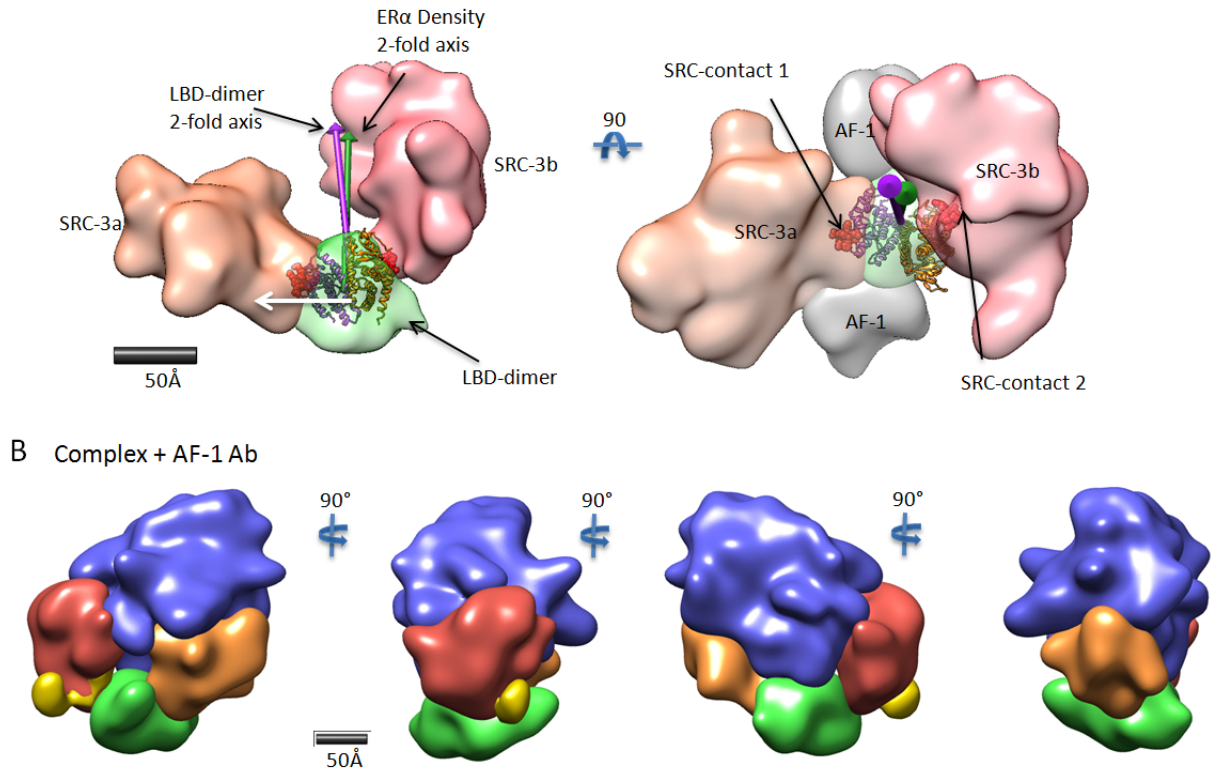

Fig. 6. Cryo-electron microscopic based representation of ERa in complex with coactivators and DNA. (A) An ERα dimer (green) is shown bound to two SRC-3 proteins (red and orange) (left). SRC-3 contacts with the ERa AF-1 are represented to the right. (B) An antibody targeting the ERα AF-1 (yellow) was used to probe the AF-1 conformation. P300 is shown in violet.

bound to the complex and to isolated p300 itself, and of ERα monoclonal antibody bound to the complex (**Fig. 6**). Further analysis identified two structurally similar and non-interacting segments as SRC-3 molecules, which bind to non-equivalent sites on one p300 and to a dimer of DNA-bound ERα. Our structural model is substantiated by various biochemical experiments, and suggests the following assembly mechanism for the complex. Each of the two ligand-bound ERα monomers independently recruits one SRC-3 molecule via the transactivation domain of ERα; the two SRC-3s in turn bind to different regions of one p300 molecule through multiple contacts. This is the first structural evidence for the location of activation domain 1 (AF-1) in a full-length nuclear receptor. This supports a role for AF-1 participation in SRC-3 recruitment.

Opportunities for training and professional development:

Nothing to report

Dissemination of results to communities of interest:
Results from work performed during the reporting period were disseminated to the research community through publications. Dr. O'Malley also presented findings from this work at the 2014 Endocrine Society Meeting in San Francisco, CA.

Plans for next reporting period:
During the next reporting period, we plan to continue our characterization of bufalin derivatives as potential improved SRC SMIs. During this time, we will investigate the ability of these derivatives to inhibit SRC transcriptional activity and promote degradation of the protein in breast cancer tumor cells. At the same time, we will investigate the effects of these derivatives on cardiac function in animals to identify compounds that lack cardiac glycoside activity. Because cardiac glycoside activity of bufalin is the key biological activity of this molecule that limits its dose *in vivo*, we anticipate the derivatives with SRC SMI activity, but lacking cardiac glycoside activity, will be more effective anti-cancer agent. We are also planning to investigate the SRC SMS, MCB-613, in greater detail and to synthesize derivatives of this compound. Already, we have found that MCB-613 can block the growth of breast cancer cells *in vitro* and we plan to test its ability to block tumor cell growth *in vivo* as well. Chemical modification of MCB-613 is much more straightforward than for bufalin, and we already have synthesized a number of derivatives that also possess biological activity. During the following year, we plan to synthesize more than 100 MCB-613 derivatives. Top derivatives will be tested in animal model systems for their ability to block breast cancer tumor growth in xenograft models. We also will determine the biodistribution of these derivatives and evaluate their toxicity *in vivo*.

4. IMPACT:
Impact on the development of the principal discipline of the project:
Our efforts to identify SMIs that can target SRCs are expected to build confidence for other researchers to develop approaches to target relatively unstructured, regulatory proteins in the future. Most drugs are designed to target enzymes or proteins with high affinity ligand binding domains (e.g. nuclear receptors). Work described here should lead to 'entirely new' approaches to target key oncoproteins that are designated as 'important but difficult' drug targets and have hitherto received little attention as for drug development.

The interpretation of our cryo-electron microscopy map has led to a model of spatial organization of all the components in this DNA-bound ERα-coactivator complex for the first time. This structural model suggests the following mechanism for the assembly of the individual components into the complex: The ERα binds the ERE DNA as a dimer, and then recruits two SRC-3 molecules; these two SRC-3 molecules, in turn, secure one molecule of p300 to the complex through multiple contacts. p300 does not bind either DNA or ERα. The cryo-electron microscopy structure of ERα-SRC-3-p300 complex we present here not only reveals the

structural organization of a transcriptionally active ERα-coactivator complex but also provides information about the overall structure of individual proteins. Using an ERα AF-1 specific antibody, we are able to visualize the position of the AF-1 function domain (A/B domain) within the full length ERα protein (Fig. 3a and b). Although several NR structures have been report, neither the location nor the structure of their AF-1 domains could be determined due to their high mobility. We observe for the first time that the A/B domain is located in proximity to the LBD (AF-2) and it also participates in the SRC-3 recruitment together with AF-2. This structural organization allows potential inter-communication between the two domains, providing a structural support for previous observations of a direct interaction between the N- and C-terminal domains and subsequent cooperative functions of these two domains in ER transactivation activity. It also may allow cooperative interaction between AF-1 and AF-2 binding coactivators.

Impact on other disciplines:
The progress made during this reporting period has advanced the state-of-the-art for the field of cryo-electron microscopy and should contribute to further efforts to apply this technique to elucidate the structures of other transcription factor complexes and to possibly understand the structural relationships between these complexes and the general transcription machinery.

Impact on technology transfer:
Nothing to report.

Impact on society beyond science and technology:
Nothing to report.

5. CHANGES/PROBLEMS:
Nothing to report.

6. PRODUCTS:
Publications:
Yan F, Yu Y, Chow DC, Palzkill T, Madoux F, Hodder P, Chase P, Griffin PR, O'Malley BW, Lonard DM. Identification of verrucarin a as a potent and selective steroid receptor coactivator-3 small molecule inhibitor. PLoS One. 2014 Apr 17;9(4):e95243. Acknowledgement of federal support: yes

Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, Wang J, Qi R, Matzuk AJ, Song X, Madoux F, Hodder P, Chase P, Griffin PR, Zhou S, Liao L, Xu J, O'Malley BW. Bufalin is a potent small-molecule inhibitor of the steroid receptor coactivators SRC-3 and SRC-1. Cancer Res. 2014 Mar 1;74(5):1506-17. Acknowledgement of federal support: yes

Dasgupta S, Lonard DM, O'Malley BW. Nuclear receptor coactivators: master regulators of human health and disease. Annu Rev Med. 2014;65:279-92. Acknowledgement of federal support: yes

Feng Q, O'Malley BW. Nuclear receptor modulation - Role of coregulators in selective estrogen receptor modulator (SERM) actions. Steroids. 2014 Jun 16. pii: S0039-128X(14)00147-0. Acknowledgement of federal support: yes

Feng Q, Zhang Z, Shea MJ, Creighton CJ, Coarfa C, Hilsenbeck SG, Lanz R, He B, Wang L, Fu X, Nardone A, Song Y, Bradner J, Mitsiades N, Mitsiades CS, Osborne CK, Schiff R, O'Malley BW. An epigenomic approach to therapy for tamoxifen-resistant breast cancer. Cell Res. 2014 Jul;24(7):809-19. Acknowledgement of federal support: yes

Website(s) or other Internet site(s):
Nothing to report.

Technologies or techniques:
Nothing to report.

Inventions, patent applications, and/or licenses:
Nothing to report.

Other Products:
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Individuals working on the project:

1. Bert W. O'Malley, M.D.: No change in role or level of effort
2. Wah Chiu, Ph.D.: No change in role or level of effort
3. Dr. Timothy Palzkill, Ph.D.: No change in role or level of effort
4. David Lonard, Ph.D.: No change in role or level of effort
5. Charles Foulds, Ph.D.: No change in role or level of effort
6. Jianming Xu, Ph.D.: No change in role or level of effort
7. Nicholas Mitsiades, M.D.,Ph.D: No change in role or level of effort
8. Yongcheng Song, Ph.D: Dr. Song is replacing Dr. Jin Wang in providing medical chemistry support in the generation of steroid receptor coactivator inhibitors at a level of 10% effort.
9. Dar-Chone Chow, Ph.D.: No change in role or level of effort
10. Fei Yan, Ph.D.: No change in role or level of effort
11. Michael T. Lewis, Ph.D.: Dr. Lewis is providing animal model support for this project in lieu of Dr. Rachel Schiff, Ph.D. 5% of Dr. Lewis' effort is supported here.
12. Lei Wang, Ph.D.: No change in role or level of effort
13. Michael Schmid, Ph.D: No change in role or level of effort
14. Zhao Wang, Ph.D.: No change in role of level of effort
13. Bokai Zhu, Ph.D.: Dr. Zhu, a postdoctoral fellow in Dr. O'Malley's laboratory, contributed 10% effort to this project, performing ChIP-Seq analyses in support of the projects goals.

14. Anil Panigrahi, Ph.D.: Dr. Panagrahi, an instructor in Dr. O'Malley's laboratory, worked at a 100% effort in this project. He is investigating estrogen receptor regulated gene expression using cell-free biolchemical approaches to support our cryo-EM goals.
15. Shrijal Shah: Mr. Shah is a research assistant hired to support Dr. Mitsiades work in this project and is contributing 100% effort here.

Changes in active other support of the PD/PI or senior/key personnel since last reporting period:
Nothing to report.

Other organizations involved as partners:
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:
Nothing to report.

9. APPENDICES:
Publications from Yan et al., Wang et al., Feng et al., and Dasgupta et al. are attached (see publication list above).

OPEN ACCESS Freely available online

PLOS ONE

Identification of Verrucarin A as a Potent and Selective Steroid Receptor Coactivator-3 Small Molecule Inhibitor

Fei Yan[1], Yang Yu[1], Dar-Chone Chow[2], Timothy Palzkill[2], Franck Madoux[3], Peter Hodder[3], Peter Chase[3], Patrick R. Griffin[3], Bert W. O'Malley[1], David M. Lonard[1]*

1 Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, United States of America, **2** Department of Pharmacology, Baylor College of Medicine, Houston, Texas, United States of America, **3** Department of Molecular Therapeutics, The Scripps Research Institute, Scripps Florida, Jupiter, Florida, United States of America

Abstract

Members of the steroid receptor coactivator (SRC) family are overexpressed in numerous types of cancers. In particular, steroid receptor coactivator 3 (SRC-3) has been recognized as a critical coactivator associated with tumor initiation, progression, recurrence, metastasis, and chemoresistance where it interacts with multiple nuclear receptors and other transcription factors to enhance their transcriptional activities and facilitate cross-talk between pathways that stimulate cancer progression. Because of its central role as an integrator of growth signaling pathways, development of small molecule inhibitors (SMIs) against SRCs have the potential to simultaneously disrupt multiple signal transduction networks and transcription factors involved in tumor progression. Here, high-throughput screening was performed to identify compounds able to inhibit the intrinsic transcriptional activities of the three members of the SRC family. Verrucarin A was identified as a SMI that can selectively promote the degradation of the SRC-3 protein, while affecting SRC-1 and SRC-2 to a lesser extent and having no impact on CARM-1 and p300 protein levels. Verrucarin A was cytotoxic toward multiple types of cancer cells at low nanomolar concentrations, but not toward normal liver cells. Moreover, verrucarin A was able to inhibit expression of the SRC-3 target genes MMP2 and MMP13 and attenuated cancer cell migration. We found that verrucarin A effectively sensitized cancer cells to treatment with other anti-cancer drugs. Binding studies revealed that verrucarin A does not bind directly to SRC-3, suggesting that it inhibits SRC-3 through its interaction with an upstream effector. In conclusion, unlike other SRC SMIs characterized by our laboratory that directly bind to SRCs, verrucarin A is a potent and selective SMI that blocks SRC-3 function through an indirect mechanism.

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* E-mail: dlonard@bcm.edu

Introduction

The p160 steroid receptor coactivator (SRC) family contains three members, SRC-1[1], SRC-2/GRIP1/TIF2 [2,3] and SRC-3/Amplified in Breast Cancer-1 [4] that interact with multiple nuclear receptors (NRs) and other transcription factors to regulate gene transcription. The N-terminus of SRCs contains a conserved bHLH-PAS (basic Helix Loop Helix-Per Arnt Sims) motif [5] involved in protein-protein interactions [6–8]. The central region of SRCs contains the NR interaction domain (RID), including three α-helical LXXLL motifs for interaction with NRs [9,10]. The C-terminal region of SRCs contains two activation domains (ADs), AD1 and AD2 that interact with other coactivators. AD1 interacts with p300/CBP while the AD2 binds to two histone methyltransferases - coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferases (PRMT1) [11–14]. The C-terminal domain of SRC-1 and SRC-3 also contains weak HAT activity [15,16].

All three SRCs have been implicated as oncoproteins. SRC-1 is overexpressed in a large subset of breast cancers and its overexpression is positively correlated with poor survival and knockdown of SRC-1 can inhibit breast cancer cell growth [17]. Other reports have implicated SRC-1 overexpression in endometrial cancer and in converting tamoxifen from an estrogen receptor-α (ERα) antagonist into an agonist [18,19]. SRC-2 overexpression has been linked to metastatic prostate cancer [20]. However, among the three SRCs, SRC-3 has been the most heavily implicated as an oncoprotein. SRC-3 overexpression has been found in multiple types of cancers, including breast [21], pancreatic [22], ovarian [23], gastric [24], prostate [25], and colorectal carcinomas [26]. High SRC-3 levels are associated with breast cancer recurrence [27] and SRC-3 overexpression is associated with tamoxifen and other endocrine therapy resistance in breast cancer patients [27–30]. Moreover, SRC-3 is associated with tumor metastasis and recurrence in gastric and liver cancers [24,31]. It is well known that SRC-3 can drive tumorigenesis by interacting with multiple NRs and other diverse transcription

factors to enhance their transcriptional activities, including the ERα [32], androgen receptor [33], progesterone receptor [34], thyroid receptor [35], AP-1, NF-κB, STAT-6, and E2F1 [17]. SRC-3 overexpression also can promote spontaneous tumor initiation and progression in an animal overexpression model system [36]. Together these findings demonstrate that SRC-3 is a key oncoprotein involved in cancer initiation, progression and metastatic growth, pointing to its importance as an important target for therapy [37].

Already, as a proof-of-principle, we characterized the small molecule compounds gossypol and bufalin as SRC small molecule inhibitors (SMIs) [38,39]. Here, a high-throughput screening assay was performed to identify improved SRC SMIs leading to the identification of verrucarin A as a potent SRC inhibitor that is structurally unrelated to gossypol or bufalin. Verrucarin A inhibits all three SRCs at higher doses, but can selectively reduce SRC-3 protein levels at lower concentrations without impacting CARM-1 or p300 protein levels. Furthermore, verrucarin A showed cytotoxic effects against various types of cancer cells but not normal liver cells, and the potencies for its cytotoxic effects are consistent with those needed to induce SRC-3 protein down regulation. Importantly, we found that verrucarin A does not detectably bind SRC-3 at its effective concentration in cell culture, implicating an upstream effector of SRC-3 as a likely target of this compound.

Materials and Methods

Chemicals, reagents and antibodies

Verrucarin A, gemcitabine, docetaxel, tamoxifen, and paclitaxel were obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO. Gefitinib and BEZ235 were purchased from Selleck Chemicals (Houston, TX, USA). Estradiol (E2) was purchased from Sigma and dissolved in ethanol. SRC-1 and SRC-3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and CARM1 and SRC-2 antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). β-actin and p300 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

All human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), cultured in DMEM (HeLa and MCF-7), RPMI-1640 (A549 and H1299), DMEM/F12 (PC-3), or MEM (HepG2), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen), in a 5% CO_2 humidified atmosphere at 37°C. Primary mouse hepatocytes were isolated as previously described [39].

1,536-well plate SRC-1 and SRC-3 HTS assays

A detailed protocol for the HTS screening assay can be found on the PubChem Bioassay website (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid = 588357&loc = ea_ras).

Cell viability assay

Cancer cell lines were plated into 96-well plates at a density of 4×10^3 cells/100 μL medium per well. After adherence, cells were treated with various concentrations of verrucarin A individually or in combination with other anti-cancer drugs for 72 h, with DMSO vehicle as a control. After treatment, relative numbers of viable cells were measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Kit (Promega, Madison, WI) according to previous described [40].

Luciferase reporter assay

Luciferase assays were performed as previously described [41]. For coactivator intrinsic activity assays, the pG5-LUC GAL4-responsive reporter plasmid (Promega) was co-transfected along with mammalian expression vectors encoding full length SRCs fused to the DNA-binding domain of GAL4 (pBIND-SRC-1, pBIND-SRC-2 and pBIND-SRC-3). For ERα/SRC coactivator assays, an estrogen-responsive reporter construct (pERE-E1b-LUC) was cotransfected along with mammalian expression vectors for ERα(pCR3.1-hERα) and SRCs (pCR3.1-SRC-1, pCR3.1-SRC-2 or pCR3.1-SRC-3). Briefly, cells were seeded in a 24-well plate and transfected with expression vectors for ERα, SRC or GAL4 DNA binding domain-SRC fusion proteins along with appropriate luciferase reporter plasmids. Twenty-four hours after transfection, cells were treated with various concentrations of verrucarin A for an additional 24 h. After treatment, cells were lysed and then luciferase activities were measured according to the manufacturer's protocol (Promega).

Immunoblot analysis

Cell lysates were loaded with equal amounts of protein onto 4–15% SDS–polyacrylamide gels, electrophoresed and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h in TBS-Tween-20 containing 5% non-fat milk and then incubated with primary antibodies at 4°C overnight. After washing, the blots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling) at room temperature for 1 h. The blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Quantitative PCR analysis

Quantitative PCR assay was performed as previously described [42]. Briefly, total RNA was isolated from cells using the RNeasy mini kit (QIAGEN, Valencia, CA, USA). RNA was converted to cDNA with a Reverse Transcription System (Promega) according to the manufacturer's instructions. Quantitative polymerase chain reaction (QPCR) transcript level determination was performed using a SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY, USA) and an ABI Prism 7700 sequence detection system (Life Technologies). The specific primers for QPCR were chosen using the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/index.html) indicated below:

5′- AATGAATACGAGCGTCTACAGC-3′

and 5′- TTTCGTCGTGTTGCCTCTTGA-3′ for SRC-1, 5′-TGGGGCCTATGATGCTTGAG-3′

and 5′- GGTTTTTGACAAATTCCGTGTGG-3′ for SRC-2, 5′- AGACGGGAGCAGGAAAGTAAA-3′

and 5′- GTAAAAGCGGTCCTAAGGAGTC-3′ for SRC-3, 5′- GGAGCGAGATCCCTCCAAAAT-3′

and 5′- GGCTGTTGTCATACTTCTCATGG-3′ for GAPDH.

The specific primers for MMP-2 and MMP-13 QPCR were from previously described [43]:

5′- TGAGCTCCCGGAAAAGATTG-3′

and 5′- TCAGCAGCCTAGCCAGTCG-3′ for MMP-2, 5′-GCAGTCTTTCTTCGGCTTAG-3′

and 5′- CAGGGTCCTTGGAGTGGTCA-3′ for MMP-13.

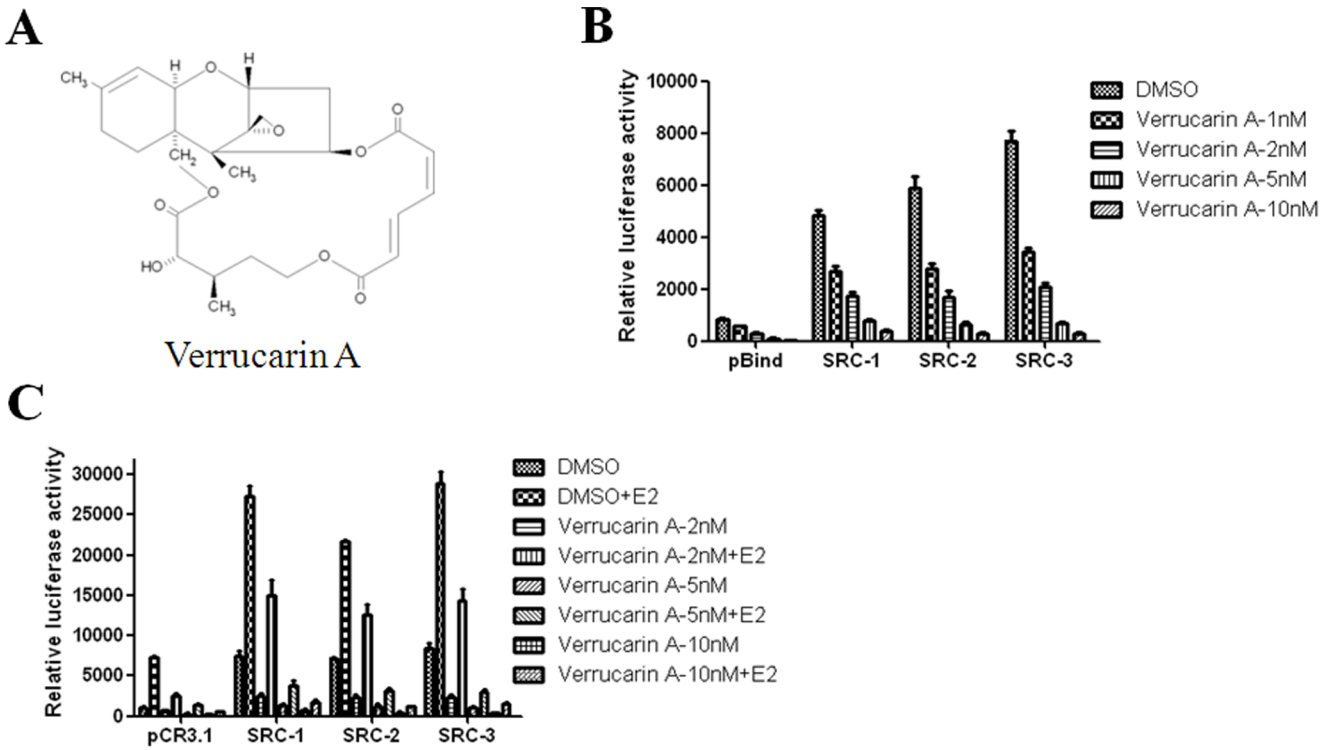

Figure 1. Verrucarin A reduces the transcriptional activities of SRCs in HeLa cells. (A) Chemical structure of verrucarin A. (B) Verrucarin A inhibits pBIND-SRC luciferase activity. HeLa cells were transiently cotransfected with expression vectors for pBIND-SRC-1, pBIND-SRC-2 or pBIND-SRC-3 and the GAL4-responsive pGL5 reporter plasmid before incubation with verrucarin A at different concentrations (0, 1, 2, 5, and 10 nM) for 24 h, followed by luciferase assay. Empty pBIND vector was transfected as a negative control. (C) Verrucarin A inhibits SRC coactivation of ERα. Luciferase assays were performed in HeLa cells transiently transfected with an ERE-luc reporter vector and expression vectors for ERα, and pCR3.1-SRC before incubation with 10 nM E2 and verrucarin A at different concentrations (0, 2, 5, and 10 nM) for 24 h.
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Fluorescence spectrometry assay

This assay was performed as previously described [39]. Briefly, the GST fusion proteins of three SRC-3 fragments were expressed and purified. Fluorescence spectrometric assays were performed using an Agilent Cary fluorescence spectrometer (Agilent Technologies Inc., Santa Clara, CA). GST-SRC-3 GST-bHLH, GST-RID, or GST-CID protein was added in a fluorescence cuvette. After addition of verrucarin A or DMSO as a negative control, the protein samples were excited by UV light at a wavelength of 278 nm with a 2-nm bandwidth, and the emission spectrum was recorded from 295 nm to above 400 nm with a bandwidth of 4 nm. The peak fluorescence intensity was 306 nm for GST-bHLH and GST-CID, and 338 nm for GST-RID. Gossypol and bufalin, two published inhibitors of SRC-3, were used as positive controls for this assay.

Wound healing assay

Cells were grown in 24-well plates to confluence and then a "wound" was created by scratching the cell monolayer using a pipette tip. Cells were treated with verrucarin A for 18 h. Photo documentation was taken at the zero time point and the 18 h time point after wounding for three independent experiments.

Statistical analysis

The Student's t-test was used to compare the significance of the differences between two groups of data. A value of $P<0.05$ was regarded as indicating a significant difference.

Results

Identification of verrucarin A as a SRC SMI

A high throughput luciferase assay-based screen of a MLPCN chemical library with 359,484 compounds [38,44] was performed to identify compounds capable of inhibiting the intrinsic transcriptional activities of SRC-3 (PubChem AID:588362), SRC-1 (PubChem AID:588354) and SRC-2 (PubChem AID:651957). Compounds were assayed by measuring luciferase expression from cells transiently transfected with a GAL4 responsive luciferase reporter (pG5-LUC) and an expression vector for either a GAL4 DNA binding (DBD) or GAL4 DBD SRC-1, SRC-2 and SRC-3 fusion proteins. Those compounds that blocked luciferase activity greater than 3σ over DMSO were scored as SMI hits. In our primary screens, the transfected HEK293 cells were treated with test compounds at a concentration of 3.6 μM in 0.36% DMSO for SRC-1 and SRC-3 or 8.9 μM for SRC-2. 3.6 μM gossypol was used as a positive control which was able to elicit 100% inhibition. Based upon a 3σ cut-off, 428 out of 359,498 (0.12%) compounds were able to inhibit SRC-1, 620 out of 359,245 (0.17%) compounds were able to inhibit SRC-2 and 621 out of 359,484 compounds inhibited SRC-3 (0.17%). A summary of SRC inhibitor screening results is shown in Table S1.

Verrucarin A was identified from these screens (Fig. 1A), belonging to a group of sesquiterpene toxins, derived from the pathogenic fungus *Myrothecium verrucaria* often found in infected food grains (Fig. 1A). Verrucarin A has been shown to be cytotoxic at sufficient levels and a variety of mechanisms have been proposed for its biological activity. As one example, it has been

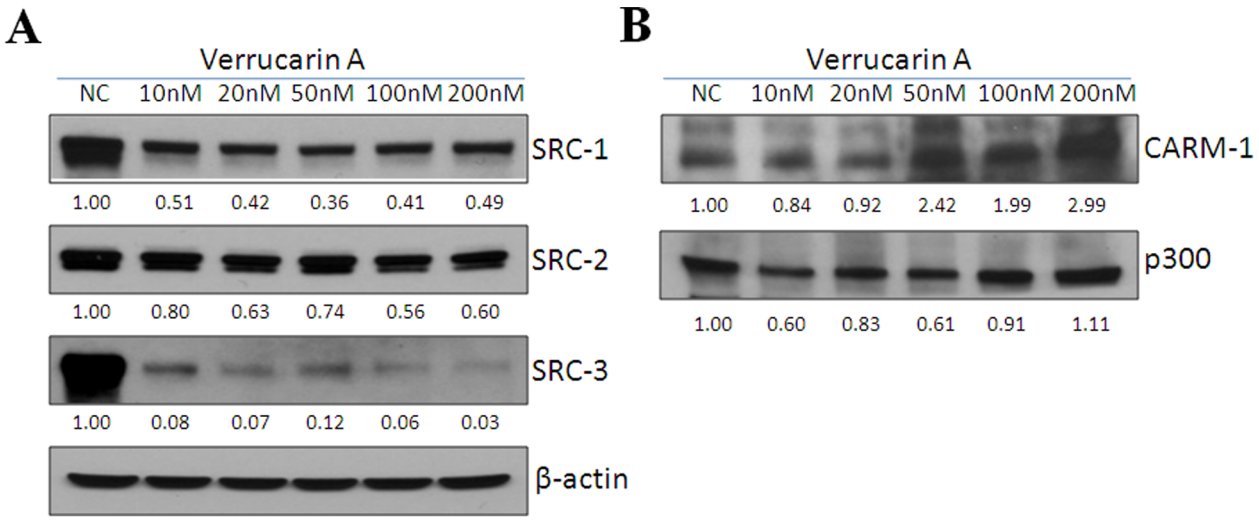

Figure 2. Verrucarin A selectively reduces SRC-3 protein levels while it does not reduce CARM-1 and p300 protein levels. (A-B) A549 cells were treated with verrucarin A at different concentrations (0, 10, 20, 50, 100, and 200 nM) for 24 h, then Western analysis was performed to quantitate SRC-1, SRC-2, SRC-3, CARM1, and p300 proteins.
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shown to inhibit protein synthesis by preventing peptidyl transferase activity [45]. Verrucarin A also can trigger a ROS-mediated intrinsic mechanism of apoptosis [46], and can induce TRAIL-induced apoptosis by eIF2α/CHOP-dependent DR5 induction via ROS generation [47]. Jayasooriya *et al.* showed that verrucarin A enhances TNF-α-induced apoptosis via NF-κB-dependent Fas overexpression [48,49]. Although, the predominant mechanism of verrucarin A-induced cell growth inhibition remains unclear, it and its derivatives are considered to be potentially useful anticancer agents [46,48,50].

To confirm the ability of verrucarin A to inhibit SRCs as seen in our primary screens, HeLa cells were transiently cotransfected with expression vectors for GAL4-DBD-SRCs (pBIND-SRCs) and a GAL4-responsive luciferase reporter (pGL5) plasmid before incubation with verrucarin A at various concentrations (0, 1, 2, 5, and 10 nM) for 24 h. Verrucarin A inhibited GAL4-responsive luciferase reporter activity of all SRCs in a dose-dependent manner (Fig. 1B). Furthermore, to evaluate SRC coactivator activities on a NR, HeLa cells were transfected with an estrogen response element (ERE) containing reporter gene and expression vectors for ERα, and SRC-1, SRC-2 and SRC-3. Twenty-four hours after transfection, cells were incubated with 10 nM E2 and verrucarin A at the indicated concentrations (0, 2, 5, and 10 nM) for 24 h followed by luciferase assays. These results indicate that verrucarin A can block SRC mediated coactivation of ERα consistent with its ability to inhibit coactivator intrinsic transcriptional activity (Fig. 1C).

Verrucarin A selectively reduces SRC-3 protein levels

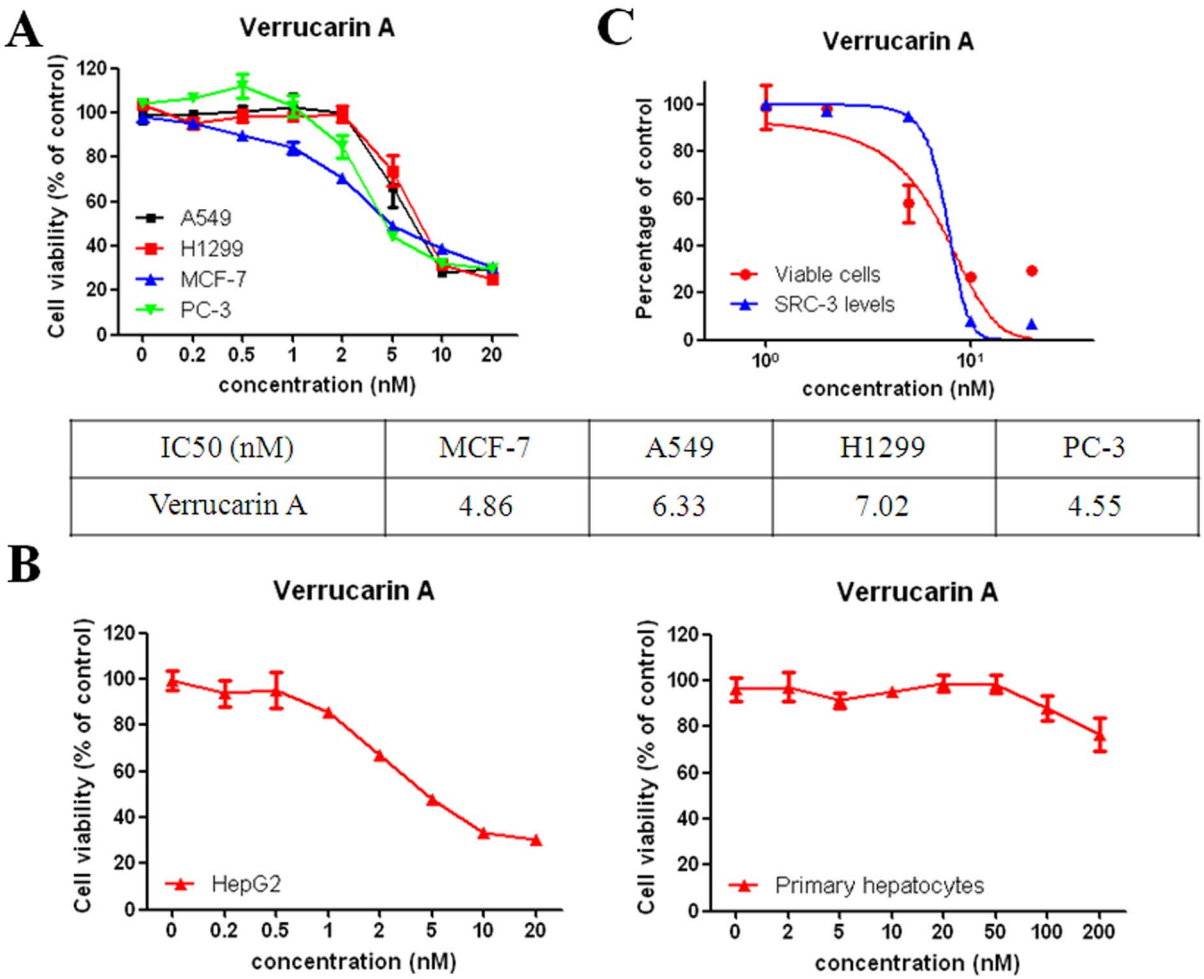
Since verrucarin A inhibits the transcriptional activities of all three SRCs, we wished to investigate if this was due to downregulation of protein expression of SRCs as we have observed for gossypol and bufalin [38,39]. A549 cells were treated with verrucarin A at various concentrations (0, 10, 20, 50, 100, and 200 nM) for 24 h, then protein levels for SRC-1, SRC-2, and SRC-3 were determined by Western analysis. Verrucarin A treatment was able to reduce SRC-3 protein expression by 90% at 10 nM, but reduced SRC-2 and SRC-1 to a smaller extent and only at much higher doses (Fig. 2A). Moreover, verrucarin A treatment did not reduce protein levels of CARM-1 and p300 (Fig. 2B). Verrucarin A also reduced SRC-3 protein levels in other cancer cells, such as LNCaP, PC-3, and MCF-7 cell lines (Fig. S1). These data suggest that verrucarin A can selectively reduce levels of SRC-3 at low nanomolar concentrations.

Verrucarin A is selectively cytotoxic to cancer cells

To test if verrucarin A-mediated downregulation of SRC-3 corresponds with cell growth inhibition, MCF-7, A549, H1299, and PC-3 cancer cells were treated with verrucarin A at different concentrations (0, 0.2, 0.5, 1, 2, 5, 10, and 20 nM) for 72 h and cell growth was determined by MTS assay. All four of these cell lines were sensitive to verrucarin A, with with IC_{50} values ranging from 4 to 8 nM (Fig. 3A). To investigate whether verrucarin A has cytotoxic effects on non-immortalized, non-transformed cells, we tested the cytotoxic effects of verrucarin A on hepatocellular HepG2 carcinoma cells and compared them against mouse primary hepatocytes. HepG2 cells and mouse primary hepatocytes were treated with verrucarin A at different concentrations for 48 h, followed by MTS assays. We found that like other cancer cell lines, HepG2 cells were sensitive to verrucarin A, with an IC_{50} value of 4.90 nM. In contrast, mouse primary hepatocytes still can survive even with verrucarin A concentrations as high as a 200 nM (Fig. 3B). Moreover, A549 cells were treated with verrucarin A at different concentrations (0, 1, 2, 5, 10, and 20 nM) for 72 h, followed by determination of SRC-3 protein levels (Fig. S2). Compared with the results of verrucarin A inhibiting A549 cell growth, verrucarin A inhibits cancer cell viability with potencies in line with its ability to downregulate SRC-3 protein levels (Fig. 3C).

Verrucarin A inhibits SRC-3 downstream target gene (MMP-2 and MMP-13) expression

Above, we demonstrated that verrucarin A reduces cellular SRC-3 protein. To investigate if verrucarin A regulates SRC-3 downstream target gene expression (MMP2 and MMP13), HeLa cells were transiently transfected with MMP2-Luc or MMP13-Luc reporter constructs and an SRC-3 expression vector before incubation with verrucarin A at different concentrations (0, 2, 5,



IC50 (nM)	MCF-7	A549	H1299	PC-3
Verrucarin A	4.86	6.33	7.02	4.55

Figure 3. Verrucarin A can selectively kill cancer, but not normal cells. (A) Verrucarin A can kill a variety of cancer cells. MCF-7, A549, H1299, and PC-3 cells were treated with verrucarin A at different concentrations (0, 0.2, 0.5, 1, 2, 5, 10, and 20 nM) for 72 h, followed by MTS assay. (B) HepG2 cells are sensitive to verrucarin A, but primary hepatocytes are not. HepG2 cells were treated with verrucarin A at different concentrations (0, 0.2, 0.5, 1, 2, 5, 10, and 20 nM) for 48 h. Primary hepatocytes were treated with verrucarin A at different concentrations (0, 2, 5, 10, 20, 50, 100, and 200 nM) for 48 h, followed by MTS assay. (C) Verrucarin A inhibits cancer cell viabilities with potencies in line with its ability to down regulate SRC-3 protein levels.
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and 10 nM) for 24 h, followed by luciferase assays [43]. Verrucarin A reduced luciferase expression driven from the MMP2 and MMP13 reporter genes (Fig. 4A). To assess the impact on endogenous MMP2 and MMP13 genes, H1299 cells were treated with verrucarin A for 24 h, then real-time PCR was performed to assess SRC-1, SRC-2, SRC-3, MMP2, and MMP13 mRNA expression. Verrucarin A inhibited MMP2 and MMP13 mRNA expression, consistent with what was observed in the reporter gene assays (Fig. 4B). Notably, the mRNA levels of for each of the three SRCs (Fig. 4C) were not downregulated, suggesting that verrucarin A reduces SRC-3 protein levels through a posttranscriptional mechanism. To explore the kinetics of verrucarin A on promoting SRC-3 protein downregulation, a time-course analysis was performed. A549 cells were treated with 20 nM verrucarin A at a series of time points (0, 0.5, 1, 2, 4, and 6 h) and then SRC-3 protein levels were examined by Western analysis. As shown in Fig. S3, a decrease in SRC-3 protein was observed 2 h after administration of verrucarin A.

Verrucarin A inhibits H1299 cell migration

Previous studies have demonstrated that SRC-3 plays important roles in tumor initiation and expansion, and functions as a critical coactivator that drives tumor cell invasion and metastasis [43,51,52]. To evaluate whether verrucarin A can block cell motility concomitantly with inhibition of SRC-3, H1299 cells were plated into 24-well plates and treated with verrucarin A for 18 h and cell motility was determined using a wound healing assay (see Materials and Methods). As shown in Fig. 5, H1299 cell migration was attenuated by verrucarin A.

Verrucarin A's SMI mechanism of action does not involve direct binding to SRC-3

Next, we sought to determine if verrucarin A physically interacts with SRC-3. We examined the ability of verrucarin A to quench the intrinsic fluorescence of different portions of the SRC-3 protein. The fluorescence emission maximum of glutathione S-transferase (GST) SRC-3 RID at 330 nm was not quenched by verrucarin A at concentrations below 10 mM which

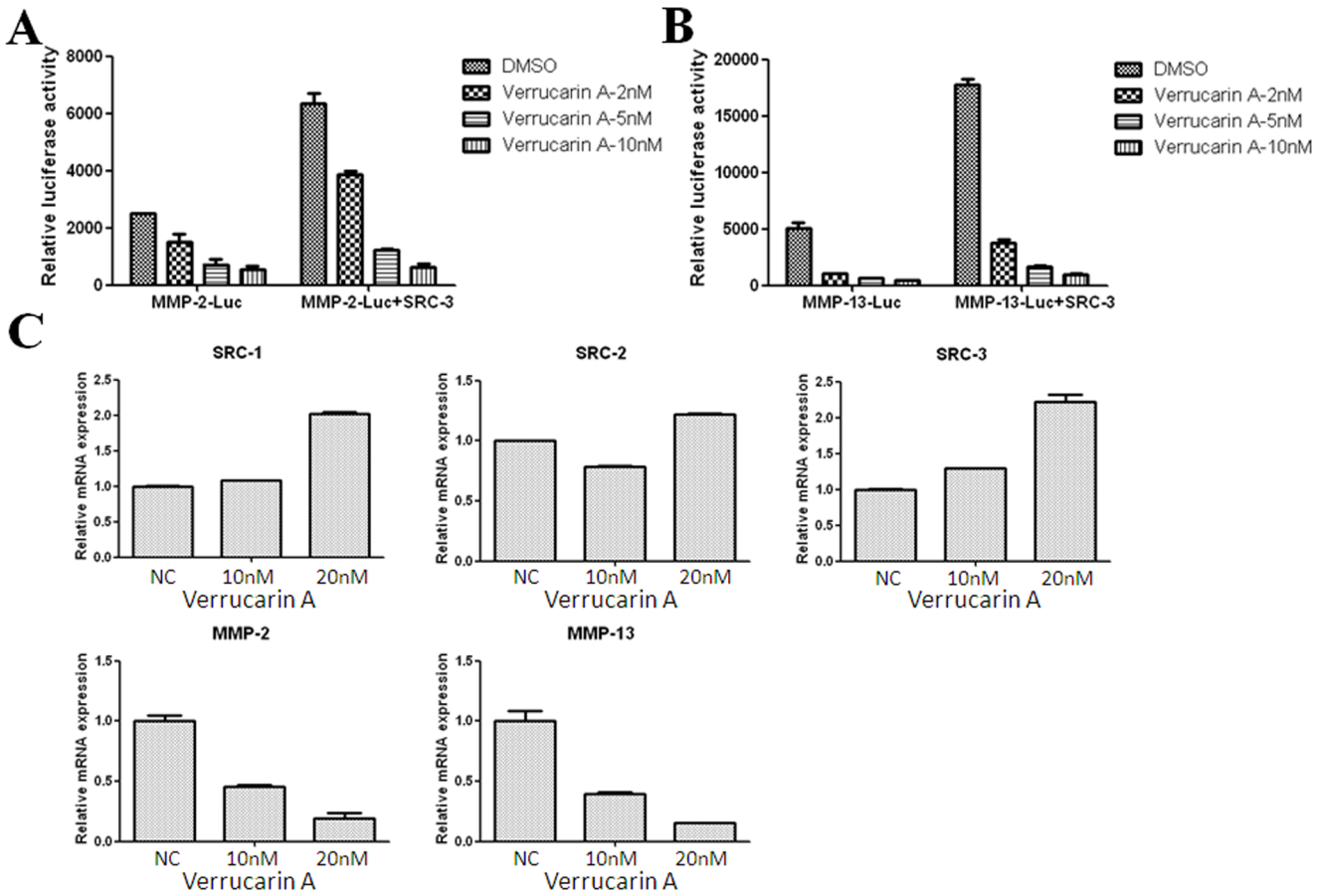

Figure 4. Verrucarin A inhibits SRC-3 downstream target gene (MMP2 and MMP13) expression. (A–B) Luciferase assays were performed in HeLa cells transiently transfected with MMP2-Luc, MMP13-Luc, and pCR3.1-SRC-3 expression vectors before incubation with verrucarin A at different concentrations (0, 2, 5, and 10 nM) for 24 h. (C) H1299 cells were treated with verrucarin A at different concentrations (0, 10, and 20 nM) for 24 h, then real-time PCR was performed to quantitate SRC-1, SRC-2, SRC-3, MMP2, and MMP13 mRNA expression.
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is well beyond its effective concentration in cell culture (data not shown). The ability of verrucarin A to quench the fluorescence of the GST SRC-3 CBP interaction domain (CID) and basic helix-loop-helix (bHLH) constructs were similarly evaluated and no nanomolar affinity binding was observed (data not shown). These data point to the likelihood that verrucarin A does not

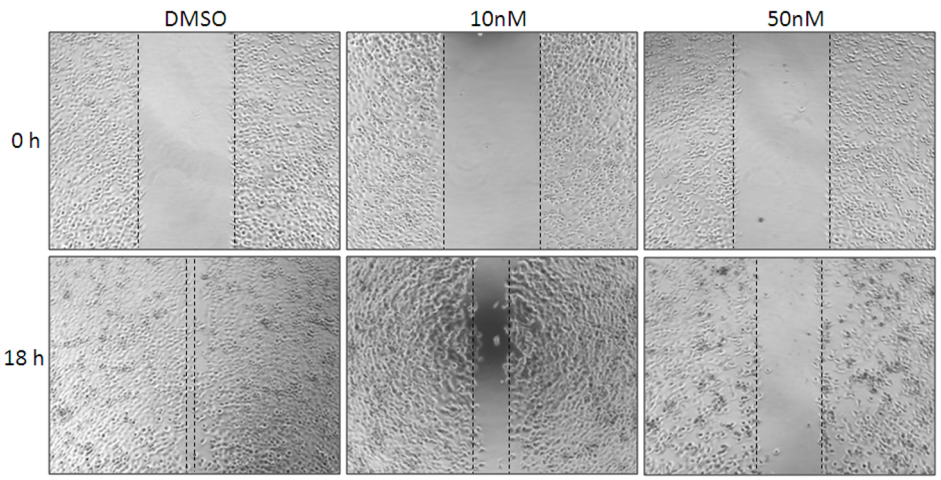

Figure 5. Verrucarin A inhibits H1299 cell migration. H1299 cells were plated into 24-well plates and treated with verrucarin A for 18 h for wound healing assay analysis.
doi:10.1371/journal.pone.0095243.g005

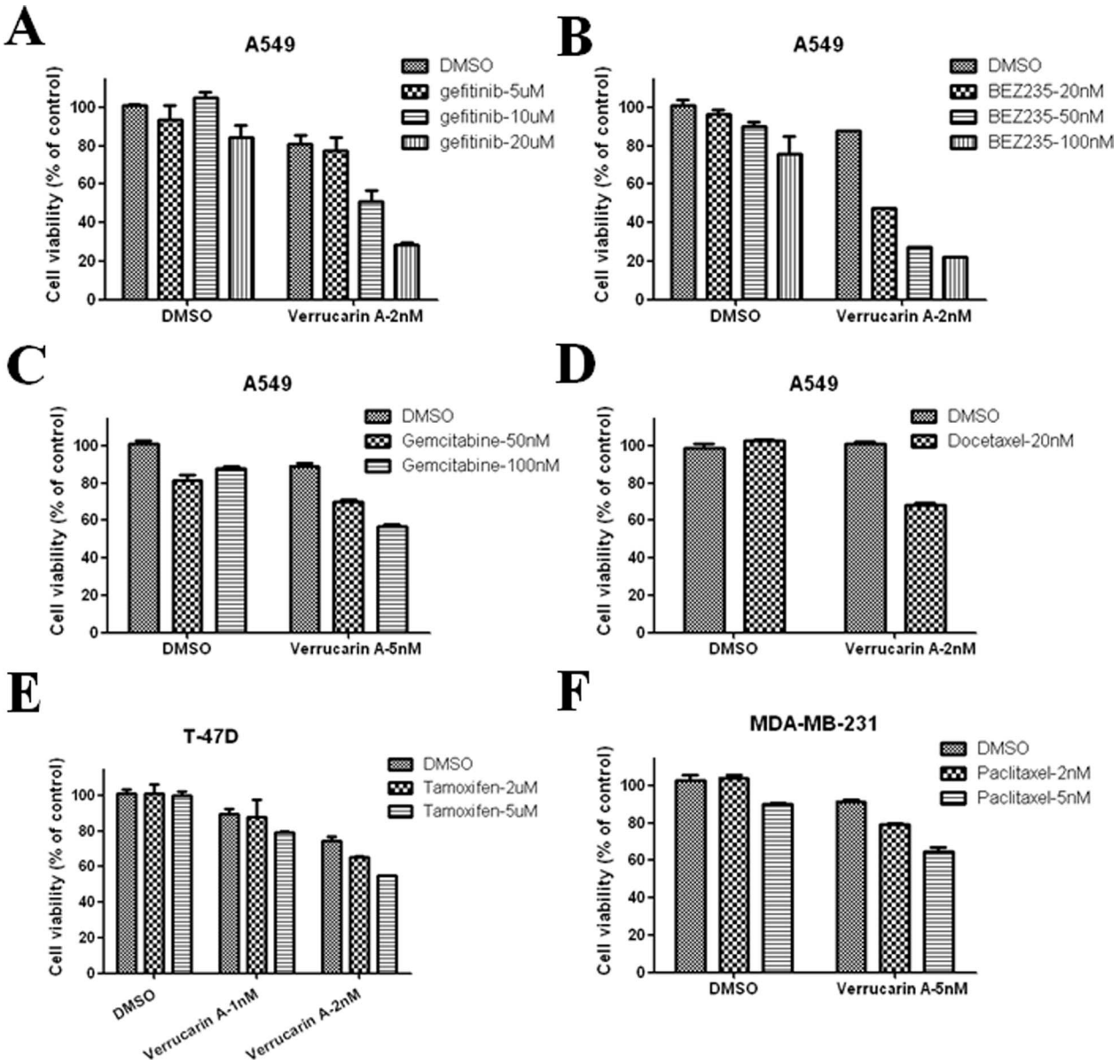

Figure 6. Verrucarin A increases cancer cell chemosensitivity to other anti-cancer drugs. (A–D) A549 cells were treated with verrucarin A in combination with gefitinib, BEZ235, gemcitabine, or docetaxel. (E) T-47D cells were treated with verrucarin A in combination with tamoxifen. (F) MDA-MB-231 cells were treated with verrucarin A in combination with paclitaxel. All cells were treated for 72 h, followed by MTS assay.
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inhibit SRC-3 by directly binding to SRC-3, but instead influences the function of an upstream mediator of SRC-3 function and protein stability.

Verrucarin A increases cancer cell chemosensitivity to other anti-cancer drugs

Numerous studies have revealed that SRC-3 is an integrator of growth promoting signaling pathways including the EGFR, HER2 and NR signaling pathways and that SRC-3 contributes to chemoresistance when it is overexpressed [30,53]. Therefore, it is expected that inhibition of SRC-3 should sensitize cancer cells to anti-cancer drugs by simultaneously blocking multiple growth signaling pathways. First, we examined the inhibitory effects of verrucarin A on cell viability in combination with four widely-used chemotherapeutic drugs (docetaxel, gemcitabine, BEZ235, and gefitinib) to block cell growth in A549 lung cancer cells. Treating A549 cells with gefitinib alone at concentrations ranging from 5 μM to 20 μM, BEZ235 from 20 nM to 100 nM, gemcitabine of 50 nM and 100 nM, or docetaxel at 20 nM had no observable effects on cell viability. However, when combined with 2 nM or 5 nM verrucarin A, the four anti-cancer drugs all inhibited cell growth in a dose-dependent manner (Fig. 6A–D). Next, we evaluated the synergistic effects of paclitaxel and tamoxifen in combination with verrucarin A on breast cancer cell viability. Exposure to a low concentration of verrucarin A significantly sensitized T-47D and MDA-MB-231 cells to tamoxifen and paclitaxel, respectively (Fig. 6E and F). These results are consistent with SRC-3′s role as an integrator of multiple growth factor signaling cascades and that its partial inhibition with verrucarin A can effectively increase cancer cell chemosensitivity to other anti-cancer drugs.

Discussion

SRC-3 overexpression has been demonstrated in a wide range of cancers as discussed above. As a coactivator for NRs and many other transcription factors, SRC-3 simultaneously drives the activity of multiple cellular signal transduction pathways. Currently, most targeted cancer drugs typically only block a single target or signaling pathway, and their clinical efficacy is frequently limited. Cancer cells typically acquire resistance to individual anticancer agents by the activation of alternative, escape growth pathways. For example, the PI3K-Akt pathways are commonly activated in ER positive breast cancer cells and can promote cell growth and confer resistance to tamoxifen [54]. However, the response of breast cancers to chronic PI3K-Akt inhibition is often limited [55], suggesting that additional growth factor escape pathways are activated. SRC-3 is a central integrator of multiple steroid hormone and hormone-independent signal transduction pathways, including the IGF-1/Akt [56,57], NF-κB [58], EGFR [59], E2F1 [60,61], and MAPK signal pathways [62,63]. Therefore, SMIs that can disrupt SRC-3 function should simultaneously prevent the activation of such a large breadth of growth pathways that underlie critical steps in cancer initiation, expansion, metastasis, and chemoresistance, that the cancer cell would be less able to overcome resistance to a SRC-3 SMI.

While the characterization of gossypol as a SRC SMI [39] validated the concept that SRCs could be targeted with a SMI, limitations in its potency provided the impetus for us to pursue high throughput screening to identify improved SRC SMIs, this ultimately led to the identification of improved SMIs such as bufalin [38] and verrucarin A. We showed that gossypol can reduce cellular protein concentrations of SRC-1 and SRC-3, without altering protein expression of SRC-2, or other coactivators, such as p300 and CARM1 [39] but only at concentrations of 5 μM which are not achievable *in vivo*. In this study, we found that verrucarin A can degrade 90% of SRC-3 protein expression at a 10 nM concentration, and about 50% of SRC-1 and SRC-2 proteins at 200 nM (Fig. 2A). Additionally, verrucarin A did not downregulate protein levels of other coactivators such as CARM-1 and p300 (Fig. 2B). Importantly, chemotherapeutic agents should be able to selectively kill tumor cells while not affecting normal cells and we found that it kills hepatocellular carcinoma HepG2 cells but not primary hepatocytes (Fig. 3B). Based on these findings, verrucarin A has promising selectivity toward tumor cells, pointing to itself and its derivatives as candidate SMIs for further development as anti-cancer agents.

In conclusion, we have identified verrucarin A as a potent, SRC-3-selecitve SMI. Clinical experience has demonstrated that combination chemotherapy regimens designed to target two distinct tumor growth pathways are often more effective than monotherapy, but even in these instances, poor response is often observed. We posit that this is due to the existence of a breadth of additional growth factor pathways available to the cancer cell. Because SRC-3 coactivates such a broad number of signaling pathways involved in cancer initiation, proliferation, motility and invasion, it stands out as a novel but promising target to combat chemoresistance. We also show that consistent with this idea, verrucarin A was able to sensitize cancer cells to a variety of established cancer drugs, highlighting the potential for SRC-3 as a key target for advanced, therapy-resistant cancers.

Supporting Information

Figure S1 Verrucarin A inhibits SRC-3 protein expression, but does not significantly reduce CARM1 and p300 protein expression in PC-3, LNCaP, and MCF-7 cells. Cells were treated with the indicated concentrations of verrucarin A for 24 h.
(TIF)

Figure S2 Verrucarin A downregulates SRC-3 protein expression in A549 cells. Cells were treated with the indicated concentrations of verrucarin A for 72 h and cell lysates were analyzed by Western blotting.
(TIF)

Figure S3 Verrucarin A induces SRC-3 protein degradation in lung cancer cells. A549 cells were treated with 20 nM verrucarin A at the indicated time points (0, 0.5, 1, 2, 4, and 6 h), and then SRC-3 protein levels were examined by Western analysis.
(TIF)

Table S1 Summary of SRC inhibitor screening.
(XLSX)

Author Contributions

Conceived and designed the experiments: FY DML BWO. Performed the experiments: FY. Analyzed the data: FY DML BWO. Contributed reagents/materials/analysis tools: YY DCC TP FM PH PC PRG. Wrote the paper: FY DML BWO.

References

1. Onate SA, Tsai SY, Tsai MJ, O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354–1357.
2. Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 15: 3667–3675.
3. Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR (1996) GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci U S A 93: 4948–4952.
4. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, et al. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277: 965–968.
5. Johnson AB, O'Malley BW (2012) Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. Mol Cell Endocrinol 348: 430–439.
6. Kim JH, Li H, Stallcup MR (2003) CoCoA, a nuclear receptor coactivator which acts through an N-terminal activation domain of p160 coactivators. Mol Cell 12: 1537–1549.
7. Belandia B, Parker MG (2000) Functional interaction between the p160 coactivator proteins and the transcriptional enhancer factor family of transcription factors. J Biol Chem 275: 30801–30805.
8. Chen SL, Dowhan DH, Hosking BM, Muscat GE (2000) The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. Genes Dev 14: 1209–1228.
9. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, et al. (1999) Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19: 8226–8239.
10. Coulthard VH, Matsuda S, Heery DM (2003) An extended LXXLL motif sequence determines the nuclear receptor binding specificity of TRAP220. J Biol Chem 278: 10942–10951.
11. Brown K, Chen Y, Underhill TM, Mymryk JS, Torchia J (2003) The coactivator p/CIP/SRC-3 facilitates retinoic acid receptor signaling via recruitment of GCN5. J Biol Chem 278: 39402–39412.
12. Liu PY, Hsieh TY, Chou WY, Huang SM (2006) Modulation of glucocorticoid receptor-interacting protein 1 (GRIP1) transactivation and co-activation activities through its C-terminal repression and self-association domains. FEBS J 273: 2172–2183.
13. Koh SS, Chen D, Lee YH, Stallcup MR (2001) Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. J Biol Chem 276: 1089–1098.

14. Ma H, Hong H, Huang SM, Irvine RA, Webb P, et al. (1999) Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol 19: 6164–6173.
15. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, et al. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90: 569–580.
16. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, et al. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389: 194–198.
17. Xu J, Wu RC, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9: 615–630.
18. Shang Y, Brown M (2002) Molecular determinants for the tissue specificity of SERMs. Science 295: 2465–2468.
19. Smith CL, Nawaz Z, O'Malley BW (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11: 657–666.
20. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, et al. (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell 18: 11–22.
21. Bautista S, Valles H, Walker RL, Anzick S, Zeillinger R, et al. (1998) In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. Clin Cancer Res 4: 2925–2929.
22. Ghadimi BM, Schrock E, Walker RL, Wangsa D, Jauho A, et al. (1999) Specific chromosomal aberrations and amplification of the AIB1 nuclear receptor coactivator gene in pancreatic carcinomas. Am J Pathol 154: 525–536.
23. Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, et al. (2000) Frequent amplification of chromosomal region 20q12–q13 in ovarian cancer. Clin Cancer Res 6: 1833–1839.
24. Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, et al. (2000) Amplification and over-expression of the AIB1 nuclear receptor co-activator gene in primary gastric cancers. Int J Cancer 89: 217–223.
25. Zhou HJ, Yan J, Luo W, Ayala G, Lin SH, et al. (2005) SRC-3 is required for prostate cancer cell proliferation and survival. Cancer Res 65: 7976–7983.
26. Xu FP, Xie D, Wen JM, Wu HX, Liu YD, et al. (2007) SRC-3/AIB1 protein and gene amplification levels in human esophageal squamous cell carcinomas. Cancer Lett 245: 69–74.
27. Mc Ilroy M, Fleming FJ, Buggy Y, Hill AD, Young LS (2006) Tamoxifen-induced ER-alpha-SRC-3 interaction in HER2 positive human breast cancer; a possible mechanism for ER isoform specific recurrence. Endocr Relat Cancer 13: 1135–1145.
28. Bouras T, Southey MC, Venter DJ (2001) Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu. Cancer Res 61: 903–907.
29. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, et al. (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96: 926–935.
30. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, et al. (2003) Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95: 353–361.
31. Wang Y, Wu MC, Sham JS, Zhang W, Wu WQ, et al. (2002) Prognostic significance of c-myc and AIB1 amplification in hepatocellular carcinoma. A broad survey using high-throughput tissue microarray. Cancer 95: 2346–2352.
32. Suen CS, Berrodin TJ, Mastroeni R, Cheskis BJ, Lyttle CR, et al. (1998) A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. J Biol Chem 273: 27645–27653.
33. Tan JA, Hall SH, Petrusz P, French FS (2000) Thyroid receptor activator molecule, TRAM-1, is an androgen receptor coactivator. Endocrinology 141: 3440–3450.
34. Han SJ, DeMayo FJ, Xu J, Tsai SY, Tsai MJ, et al. (2006) Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. Mol Endocrinol 20: 45–55.
35. Ying H, Furuya F, Willingham MC, Xu J, O'Malley BW, et al. (2005) Dual functions of the steroid hormone receptor coactivator 3 in modulating resistance to thyroid hormone. Mol Cell Biol 25: 7687–7695.
36. Torres-Arzayus MI, Zhao J, Bronson R, Brown M (2010) Estrogen-dependent and estrogen-independent mechanisms contribute to AIB1-mediated tumor formation. Cancer Res 70: 4102–4111.
37. Lydon JP, O'Malley BW (2011) Minireview: steroid receptor coactivator-3: a multifarious coregulator in mammary gland metastasis. Endocrinology 152: 19–25.
38. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, et al. (2014) Bufalin is a potent small molecule inhibitor of the steroid receptor coactivators SRC-3 and SRC-1. Cancer Res.
39. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, et al. (2011) Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. Mol Endocrinol 25: 2041–2053.
40. Yan F, Cao XX, Jiang HX, Zhao XL, Wang JY, et al. (2010) A novel water-soluble gossypol derivative increases chemotherapeutic sensitivity and promotes growth inhibition in colon cancer. J Med Chem 53: 5502–5510.
41. Wu S, Chen L, Becker A, Schonbrunn E, Chen J (2012) Casein kinase 1alpha regulates an MDMX intramolecular interaction to stimulate p53 binding. Mol Cell Biol 32: 4821–4832.
42. Wu SF, Huang Y, Hou JK, Yuan TT, Zhou CX, et al. (2010) The downregulation of onzin expression by PKCepsilon-ERK2 signaling and its potential role in AML cell differentiation. Leukemia 24: 544–551.
43. Yan J, Erdem H, Li R, Cai Y, Ayala G, et al. (2008) Steroid receptor coactivator-3/AIB1 promotes cell migration and invasiveness through focal adhesion turnover and matrix metalloproteinase expression. Cancer Res 68: 5460–5468.
44. Roy A, McDonald PR, Sittampalam S, Chaguturu R (2010) Open access high throughput drug discovery in the public domain: a Mount Everest in the making. Curr Pharm Biotechnol 11: 764–778.
45. Jimenez A, Vazquez D (1975) Quantitative binding of antibiotics to ribosomes from a yeast mutant altered on the peptidyl-transferase center. Eur J Biochem 54: 483–492.
46. Palanivel K, Kanimozhi V, Kadalmani B, Akbarsha MA (2013) Verrucarin A, a protein synthesis inhibitor, induces growth inhibition and apoptosis in breast cancer cell lines MDA-MB-231 and T47D. Biotechnol Lett 35: 1395–1403.
47. Moon DO, Asami Y, Long H, Jang JH, Bae EY, et al. (2013) Verrucarin A sensitizes TRAIL-induced apoptosis via the upregulation of DR5 in an eIF2alpha/CHOP-dependent manner. Toxicol In Vitro 27: 257–263.
48. Jayasooriya RG, Moon DO, Yun SG, Choi YH, Asami Y, et al. (2013) Verrucarin A enhances TRAIL-induced apoptosis via NF-kappaB-mediated Fas overexpression. Food Chem Toxicol 55: 1–7.
49. Jayasooriya RG, Moon DO, Park SR, Choi YH, Asami Y, et al. (2013) Combined treatment with verrucarin A and tumor necrosis factor-alpha sensitizes apoptosis by overexpression of nuclear factor-kappaB-mediated Fas. Environ Toxicol Pharmacol 36: 303–310.
50. Jarvis BB, Stahly GP, Curtis CR (1978) Antitumor activity of fungal metabolites: verrucarin beta-9, 10-epoxides. Cancer Treat Rep 62: 1585–1586.
51. Qin L, Liao L, Redmond A, Young L, Yuan Y, et al. (2008) The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. Mol Cell Biol 28: 5937–5950.
52. Li LB, Louie MC, Chen HW, Zou JX (2008) Proto-oncogene ACTR/AIB1 promotes cancer cell invasion by up-regulating specific matrix metalloproteinase expression. Cancer Lett 261: 64–73.
53. Lonard DM, Lanz RB, O'Malley BW (2007) Nuclear receptor coregulators and human disease. Endocr Rev 28: 575–587.
54. Lonard DM, O'Malley BW (2012) Nuclear receptor coregulators: modulators of pathology and therapeutic targets. Nat Rev Endocrinol 8: 598–604.
55. Ciruelos E, Cortes-Funes H, Ghanem I, Manso L, Arteaga C (2013) Role of inhibitors of mammalian target of rapamycin in the treatment of luminal breast cancer. Anticancer Drugs 24: 769–780.
56. Liao L, Chen X, Wang S, Parlow AF, Xu J (2008) Steroid receptor coactivator 3 maintains circulating insulin-like growth factor I (IGF-I) by controlling IGF-binding protein 3 expression. Mol Cell Biol 28: 2460–2469.
57. Yan J, Yu CT, Ozen M, Ittmann M, Tsai SY, et al. (2006) Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. Cancer Res 66: 11039–11046.
58. Wu RC, Qin J, Hashimoto Y, Wong J, Xu J, et al. (2002) Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. Mol Cell Biol 22: 3549–3561.
59. Long W, Yi P, Amazit L, LaMarca HL, Ashcroft F, et al. (2010) SRC-3Delta4 mediates the interaction of EGFR with FAK to promote cell migration. Mol Cell 37: 321–332.
60. Louie MC, Zou JX, Rabinovich A, Chen HW (2004) ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. Mol Cell Biol 24: 5157–5171.
61. Mussi P, Yu C, O'Malley BW, Xu J (2006) Stimulation of steroid receptor coactivator-3 (SRC-3) gene overexpression by a positive regulatory loop of E2F1 and SRC-3. Mol Endocrinol 20: 3105–3119.
62. Long W, Foulds CE, Qin J, Liu J, Ding C, et al. (2012) ERK3 signals through SRC-3 coactivator to promote human lung cancer cell invasion. J Clin Invest 122: 1869–1880.
63. Gianni M, Parrella E, Raska I, Jr., Gaillard E, Nigro EA, et al. (2006) P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RARalpha-mediated transcription. EMBO J 25: 739–751.

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Cancer Research

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Therapeutics, Targets, and Chemical Biology

Cancer Research

Bufalin Is a Potent Small-Molecule Inhibitor of the Steroid Receptor Coactivators SRC-3 and SRC-1

Ying Wang[1], David M. Lonard[1], Yang Yu[1], Dar-Chone Chow[2], Timothy G. Palzkill[2], Jin Wang[2], Ruogu Qi[2], Alexander J. Matzuk[2], Xianzhou Song[2], Franck Madoux[3], Peter Hodder[3], Peter Chase[3], Patrick R. Griffin[3], Suoling Zhou[1], Lan Liao[1], Jianming Xu[1], and Bert W. O'Malley[1]

Abstract

Virtually all transcription factors partner with coactivators that recruit chromatin remodeling factors and interact with the basal transcription machinery. Coactivators have been implicated in cancer cell proliferation, invasion, and metastasis, including the p160 steroid receptor coactivator (SRC) family composed of SRC-1 (NCOA1), SRC-2 (TIF2/GRIP1/NCOA2), and SRC-3 (AIB1/ACTR/NCOA3). Given their broad involvement in many cancers, they represent candidate molecular targets for new chemotherapeutics. Here, we report on the results of a high-throughput screening effort that identified the cardiac glycoside bufalin as a potent small-molecule inhibitor for SRC-3 and SRC-1. Bufalin strongly promoted SRC-3 protein degradation and was able to block cancer cell growth at nanomolar concentrations. When incorporated into a nanoparticle delivery system, bufalin was able to reduce tumor growth in a mouse xenograft model of breast cancer. Our work identifies bufalin as a potentially broad-spectrum small-molecule inhibitor for cancer. *Cancer Res; 74(5); 1506–17. ©2014 AACR.*

Introduction

The steroid receptor coactivator (SRC) family comprises three members, SRC-1 (NCOA1; ref. 1), SRC-2 (NCOA2; refs. 2, 3), and SRC-3 (NCOA3; refs. 4–7). Numerous studies have established broad molecular and physiologic roles for the SRC family in activating nuclear receptor–mediated gene expression (8). Besides nuclear receptors, they also coactivate other transcriptional factors, including NF-κB (NFKB1)-, E2F1-, and insulin-like growth factor I (IGF-I)–dependent transcriptional factors (9–11). The expansive roles that they play in mediating gene expression is consistent with a breadth of studies pointing to their regulation of diverse physiologic and pathophysiologic processes, including cancers in which they are frequently overexpressed (8).

Among the SRCs, SRC-3 has been the family member most prominently linked to a wide variety of cancers. SRC-3 overexpression in the mouse mammary gland leads to spontaneous tumor formation (12). In contrast, loss of SRC-3 suppressed oncogene- and carcinogen-induced breast cancer initiation, progression, and metastasis in mouse tumor models (13, 14). In humans, SRC-3 gene amplification has been found in 9.5% breast cancers and the mRNA for SRC-3 is overexpressed 64% of the time (4). Clinical and preclinical studies have shown that overexpression of SRC-3 and SRC-1 is linked to resistance to endocrine therapies in breast cancers. For instance, high expression of SRC-3, especially conjunction with high levels of EGF receptor (EGFR) and HER2 (ERBB2), is associated with poor outcome and recurrence after tamoxifen treatment (15). In ERBB2-overexpressing breast cancer cells, overexpression of SRC-3 also contributes to resistance against the ERBB2-targeting drug trastuzumab (Herceptin; ref. 16). SRC-3 overexpression has also been observed in a wide range of other cancers, including ovarian (17), endometrial (18), prostate (19, 20), liver (21), pancreatic (22), colorectal (23), and lung cancers (24).

SRC-1 has also been clearly implicated in cancer progression. In a mouse mammary tumor virus polyoma middle T (MMTV-PyMT) model system, loss of SRC-1 markedly reduces tumor cell metastasis (25). Consistent with this, elevated SRC-1 expression has been reported in approximately 20% of primary human breast cancers, with higher expression positively correlating with disease recurrence and poor survival (26, 27). A positive association between high SRC-1 expression and tumor recurrence in patients with breast cancer who received tamoxifen therapy has also been reported (26, 27).

Considering the ability of SRC coactivators to activate multiple growth factor signaling pathways that drive cancer cell growth and promote resistance to endocrine therapy, SRC small-molecule inhibitors (SMI) are anticipated to be effective new agents to treat cancer. In an initial "proof-of-principle" study, we recently showed that gossypol is able to inhibit SRC-3 and SRC-1 (28), demonstrating that these oncogenic coactivators are a class of accessible targets for SMI-based chemotherapy. This work established the feasibility for engaging in a high-throughput compound library screen to identify more

Authors' Affiliations: Departments of [1]Molecular and Cellular Biology and [2]Pharmacology, Baylor College of Medicine, Houston, Texas; and [3]Department of Molecular Therapeutics, The Scripps Research Institute, Scripps Florida, Jupiter, Florida

Y. Wang and D.M. Lonard contributed equally to this work.

Corresponding Authors: David M. Lonard, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: 713-798-6258; Fax: 713-790-1275; E-mail: dlonard@bcm.edu; and Bert W. O'Malley, berto@bcm.edu

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effective SRC SMIs. As a result of these high-throughput screens, here we describe the characterization of the cardiac glycoside bufalin as potent SRC SMI that is able to effectively reduce SRC-3 and SRC-1 cellular protein concentration and block cancer cell growth in cell culture and animal models.

Materials and Methods

Chemicals

For high-throughput screening, an MLSMR library was provided by Evotec through the Roadmap Molecular Libraries Initiative of the NIH. Details about compound selection for this library can be found online (29). Digoxin, bufalin, ouabain, and digitoxin were obtained from Sigma and dissolved in ethanol. Cinobufagin, cinobufotalin, cycloheximide, and MG132 were obtained from Sigma and dissolved in dimethyl sulfoxide (DMSO). Strophanthidin and resibufogenin were purchased from Santa Cruz Biotechnology and dissolved in ethanol and DMSO, respectively. MK-2206 was purchased from Selleck-Chem and dissolved in DMSO. Antibodies to SRC-1, SRC-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology. Antibodies to CARM1 and SRC-2 were obtained from Bethyl Laboratories.

Cell culture

Human cancer cell lines were obtained from the American Type Culture Collection (ATCC). Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; HeLa; MCF-7) and RPMI-1640 (A549) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin (100 U/mL), unless otherwise indicated. All cells were cultured at 37°C under 5% CO_2. All cells were used within 6 months from the time when they were obtained from ATCC, expanded, and resuscitated except for MDA-MB-231-LM3.3, which were verified to be derived from MB-MBA-231 cells by short tandem repeat DNA profiling before use in xenograft animal model experiments.

Plasmids and transfections

The expression vectors for the GAL4-responsive luciferase reporter pG5-LUC and Gal4 DBD fusion proteins with SRC-1, SRC-2, and SRC-3 were described previously (30). Twenty-four

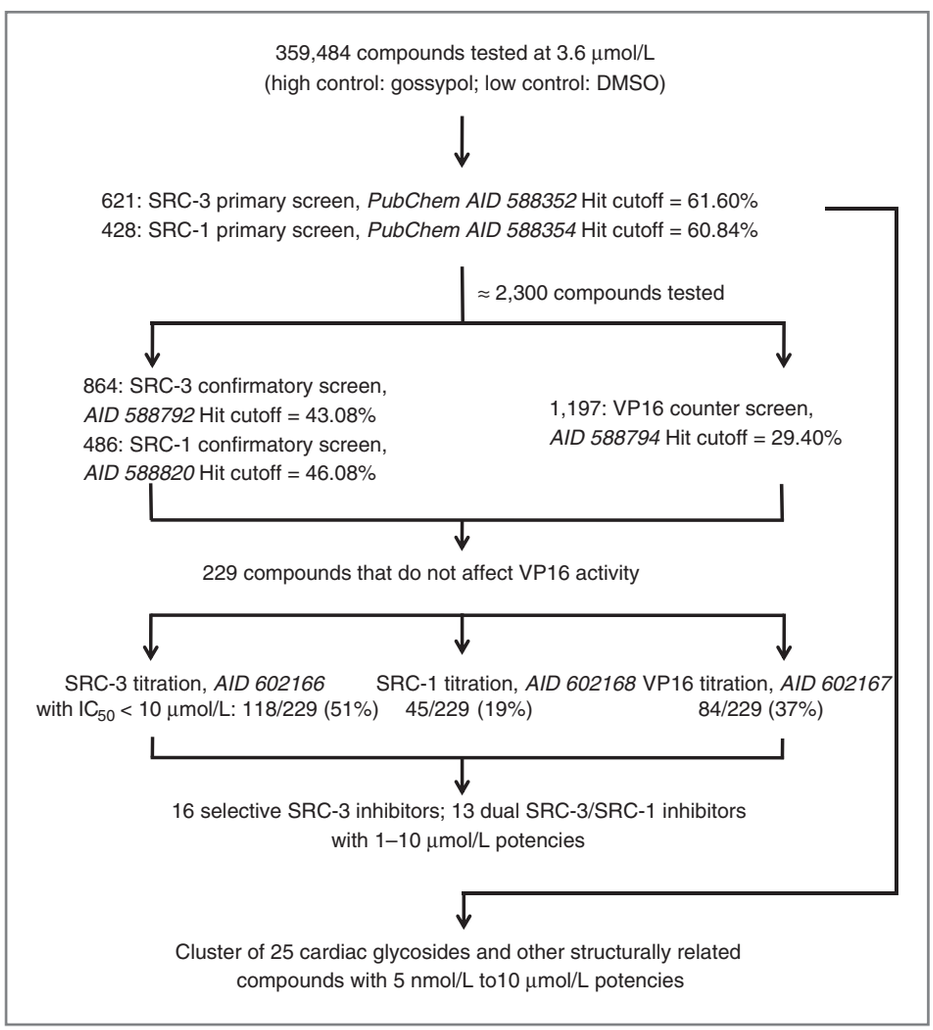

Figure 1. A flowchart of the high-throughput screen to identify inhibitors targeting SRC-3 and SRC-1. The PubChem ID for each individual assay (AID) has been indicated and summary results from these assays have been deposited to the PubChem database (50).

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hours before transfection, HeLa cells were plated in 24-well dishes. Cells were transfected with the indicated expression vector plasmids using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol before incubation with chemicals at the indicated concentrations.

Cell extraction and assays

For luciferase assays, collected cell pellets after treatments were lysed and assayed for luciferase activity. Luciferase activities were normalized against *Renilla* luciferase activities according to the manufacturer's recommendations (Promega). For Western blotting, cells were harvested and lysed in lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.5% NP-40) and then centrifuged for 15 minutes at 21,000 × *g* at 4°C. After the total cellular protein concentration was determined by Bradford analysis (Bio-Rad), protein lysates were resolved by SDS-7.5% PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked and incubated with indicated antibodies as previously described (28). All experiments were repeated at least three times. Intensities of the bands of interest in the Western blot analysis were quantitated using ImageJ software (31).

1,536-well plate SRC-1 and SRC-3 HTS assays

A detailed protocol for the high throughput screening (HTS) assay can be found on the PubChem Bioassay website (32).

Quantitative PCR analysis

MCF-7 cell total RNAs were isolated from 12-well culture dishes using the RNeasy Mini Kit (Qiagen). The mRNAs for SRC-1, SRC-2, SRC-3, and GAPDH were quantitated by TaqMan-based reverse transcriptase PCR (RT-PCR) using the ABI Prism 7700 sequence detection system (Applied Biosystems). For SRC-1, the primer pair 5′-gcaaccagctctcatccact-3′ and 5′-

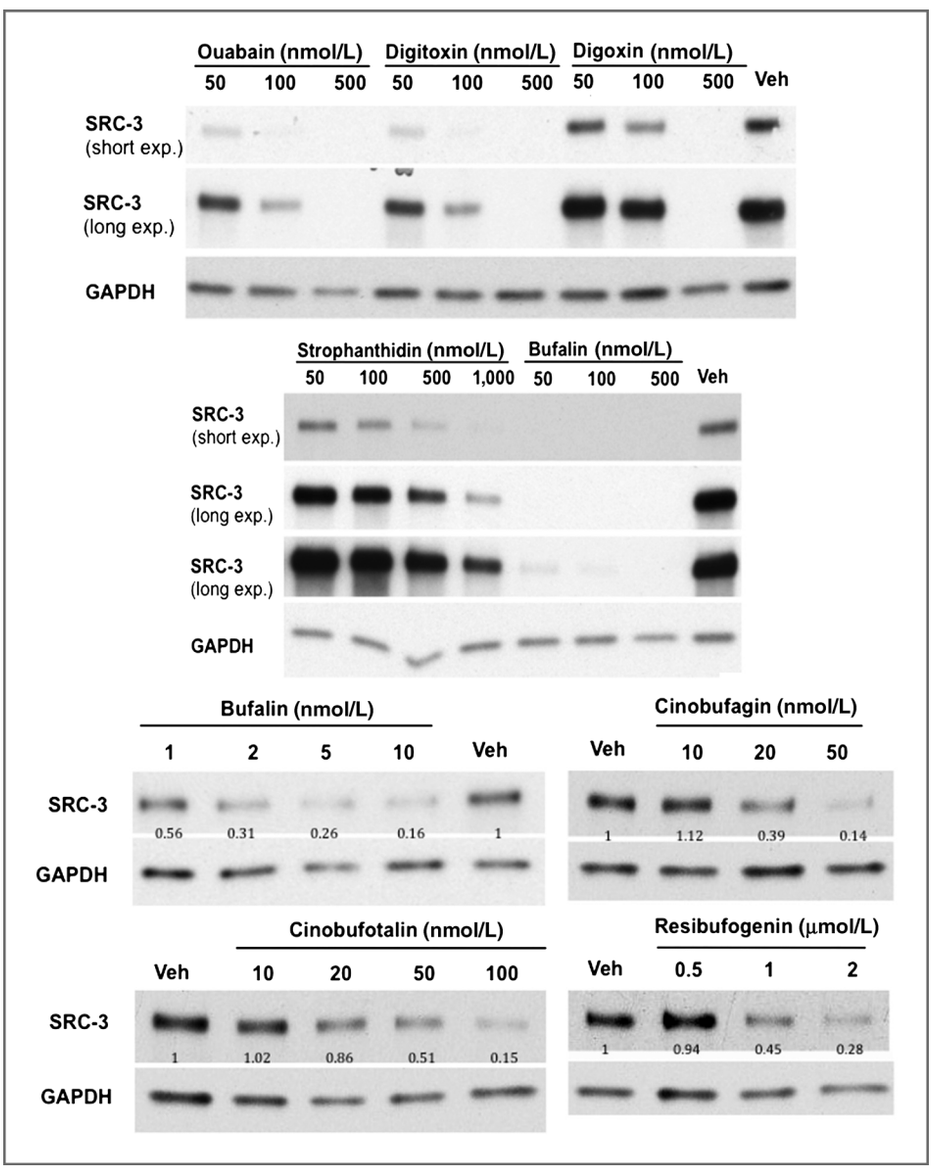

Figure 2. A panel of cardiac glycosides can reduce cellular SRC-3 protein concentrations in MCF-7 breast cancer cells. Cells were treated with plant-derived cardiac glycosides (ouabain, digitoxin, digoxin, and strophanthidin) and Asiatic toad–derived cardiac glycosides (bufalin, cinobufagin, cinobufotalin, and resibufogenin) at increasing doses for 24 hours, then SRC-3 protein levels were determined by Western blot analysis.

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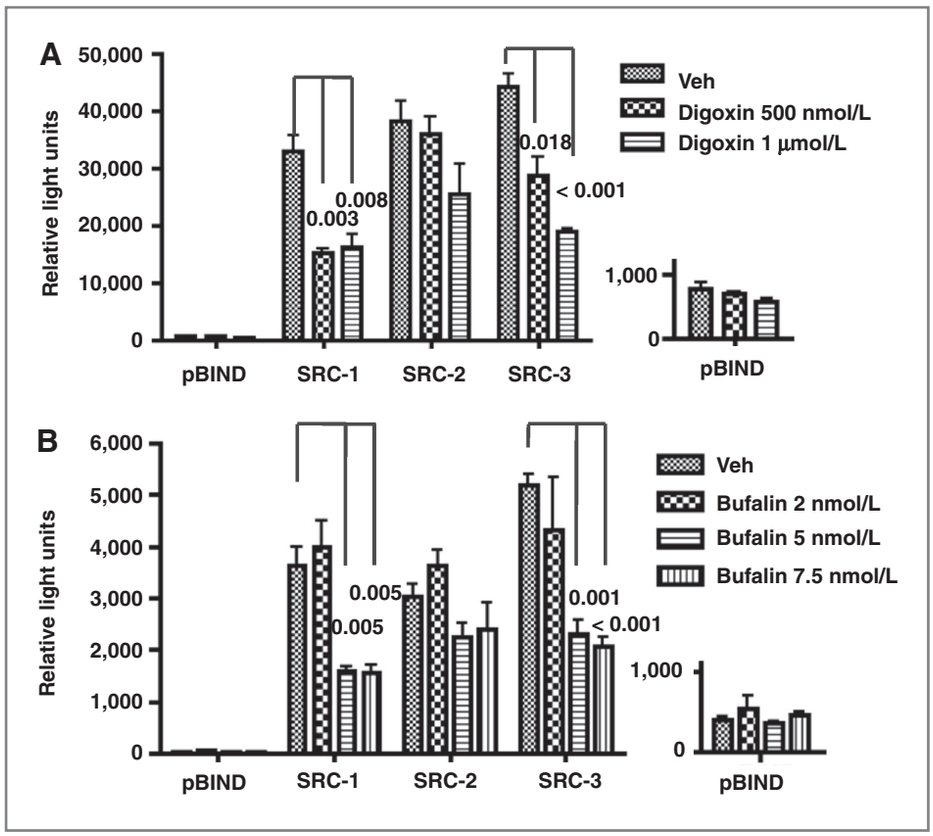

Figure 3. Digoxin and bufalin can selectively reduce the intrinsic transcriptional activities of SRC-1 and SRC-3. Luciferase assays were performed in HeLa cells transiently transfected with the reporter vector pG5-LUC in combination with expression vectors for pBIND, pBIND SRC-1, SRC-2, or SRC-3 before incubation with digoxin (0, 500 nmol/L, and 1 μmol/L; A) and bufalin (B) of different doses (0, 2, 5, and 7.5 nmol/L) for 24 hours. The difference between the vehicle-treated control and compound-treated samples was analyzed for significance by a Student *t* test, with the number of *P* value indicated above the column. Only *P* values less than 0.05 are shown in the chart. Insets on the right show the outputs with the empty vector pBIND.

gacgtcagcaaacacctgaa-3′ was used along with Universal Roche Probe #3 (Roche). For SRC-2, the primer pair 5′-aggcaacctgttcc-caaac-3′ and 5′-actggcttcagcagtgtcag-3′ was used along with Universal Roche Probe #27. For SRC-3, the primer pair 5′-agctgagctgcgaggaaa-3′ and 5′-gagtccaccatccagcaagt-3′ was used with Universal Roche Probe #70. For GAPDH, the primer pair 5′-agccacatcgctcagacac-3′ and 5′-gcccaatacgaccaaatcc-3′ was used along with Universal Roche Probe #60. RT-PCR was performed using 1× TaqMan Universal PCR Master Mix (Applied Biosystems). All mRNA quantities were normalized against GAPDH RNA and experiments were repeated two times.

Fluorescence spectrometry

The glutathione *S*-transferase (GST) fusion proteins of different portions of SRC-3 were expressed and purified as described previously (33). Fluorescence spectrometric measurements were performed using an SLM 48000S fluorescence spectrophotometer (SLM-Aminco) and an Agilent Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Inc.). A total of 1.5 μmol/L of GST SRC-3 receptor-interacting domain (RID), CBP-interacting domain (CID), or basic helix–loop–helix (bHLH) was placed in a fluorescence cuvette and excited with UV light at a wavelength of 278 nm with a 2-nm bandwidth, and the emission spectra were recorded from 295 nm to more than 400 nm with a bandwidth of 4 nm. The aliquot size of test compound was maintained below 5% of the total sample volume to minimize the effects of dilution.

Cell viability assays

Cells were seeded in 96-well plates in medium supplemented with 10% FCS and allowed to reach 70% to 80% confluence, while relative numbers of viable cells were measured with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) after compound treatment. IC_{50} values were determined using the Prism 4 software (GraphPad Software, Inc.).

Bufalin PLGA nanoparticles

A block copolymer of polylactic-co-glycolic acid (PLGA) and polyethylene glycol (PEG) was chosen as a nanocarrier material. PLGA–PEG was synthesized and bufalin-containing nanoparticles were produced using a nanoprecipitation method. Specifically, a mixture of bufalin and PLGA–PEG in trifluoroethanol (TFE) was dripped into water under constant stirring. The formed bufalin nanoparticles were verified for proper formation by dynamic light scattering (DLS) and transmission electron micrography (TEM). The amount of drug encapsulated in nanoparticles was determined to be 1% by weight using high-performance liquid chromatography (HPLC) followed by detection of absorption at a wavelength of 210 nm.

MDA-MB-231-LM3.3 xenograft tumor model

Six- to 7-week-old severe combined immunodeficient mice (SCID) mice were obtained from Charles River Laboratories. Of note, 750,000 MDA-MB-231-LM3.3 cells were injected into one of the second mammary fat pads on day 1 with tumors becoming palpable in 6 days. At this time, animals were treated

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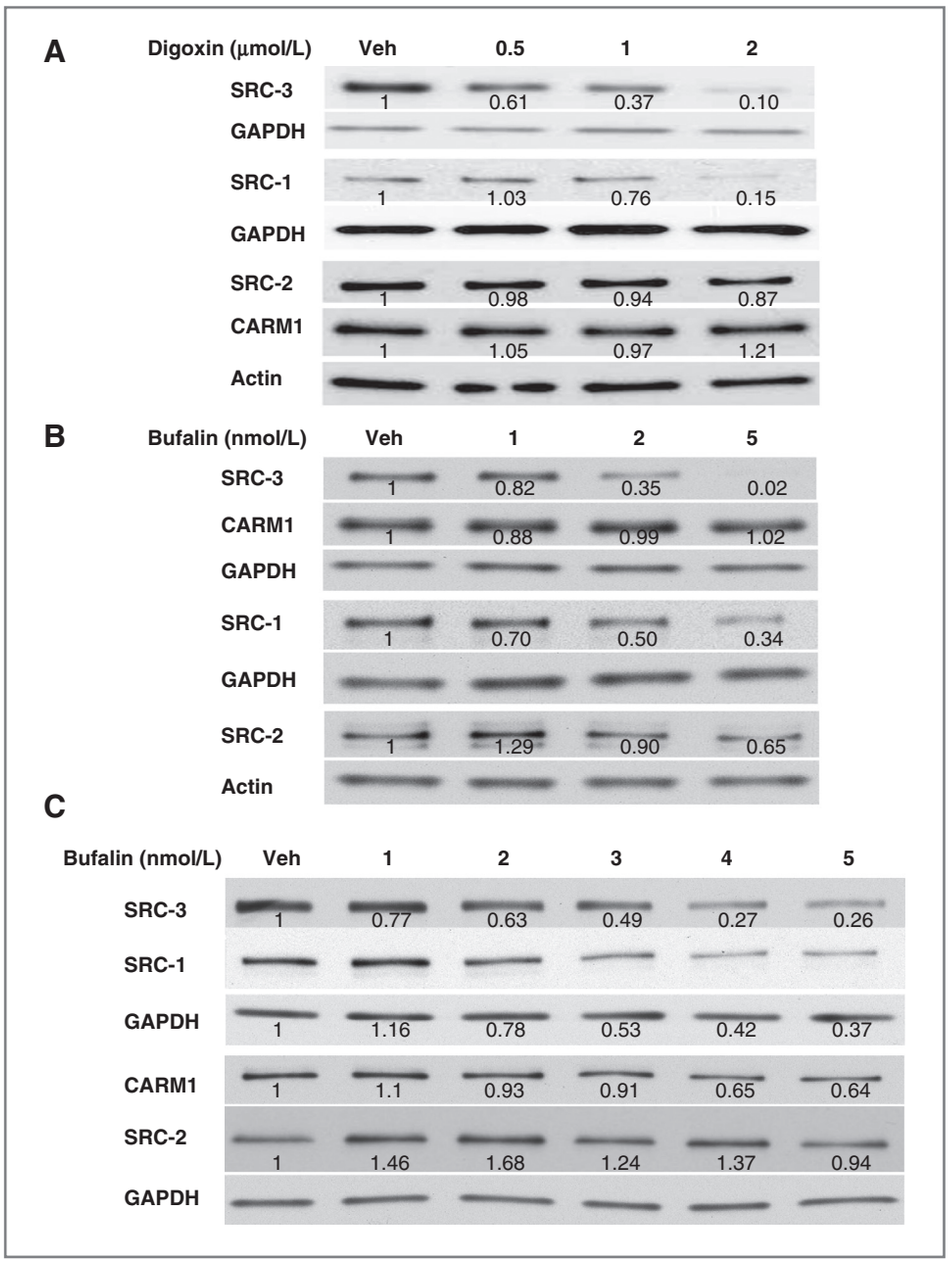

Figure 4. Digoxin and bufalin selectively reduce cellular protein levels of SRC-3 and SRC-1, but not that of SRC-2. A, MCF-7 cells were treated with digoxin (0, 0.5, 1, and 2 μmol/L) for 24 hours. Cell extracts were then blotted and probed using antibodies against SRC-1, SRC-2, SRC-3, CARM1, actin, and GAPDH. B and C, A549 cells were treated with bufalin (0, 1, 2, and 5 nmol/L; B) and MCF-7 cells (C) were treated with bufalin (0, 1, 2, 3, 4, and 5 nmol/L), and analyzed as described in A. Relative intensities of bands were normalized by either GAPDH or by actin as shown.

with either PBS, empty nanoparticles, or bufalin-loaded nanoparticles three times per week. Tumor length and width were measured daily, with volume estimated using the formula (length × width × height)/2.

Results

High-throughput screening of SRC-3 and SRC-1 inhibitors

Through a high-throughput luciferase assay–based screen, a MLPCN chemical library containing 359,484 compounds (34) was screened to identify compounds capable of inhibiting the intrinsic transcriptional activities of SRC-3 (PubChem AID:588362) and SRC-1 (PubChem AID:588354; Fig. 1). Here, compounds were evaluated by measuring luciferase expression from cells transiently transfected with a GAL4 responsive luciferase reporter (pG5-LUC) and an expression vector for either a GAL4 DNA-binding (DBD) SRC-3 or GAL4 DBD SRC-1 fusion protein (30). Compounds that inhibited luciferase gene expression greater than 3σ over DMSO were counted as SMI hits. In this primary screen, the transfected HEK293 cells were treated with test compounds at a concentration of 3.6 μmol/L in 0.36% DMSO. Of note, 36 μmol/L gossypol was used as a positive control, which was able to elicit 100% inhibition. On the basis of a 3-σ cutoff, 620 (0.17%) compounds were able to inhibit SRC-3 and 428 (0.12%) compounds were able to inhibit SRC-1. These active

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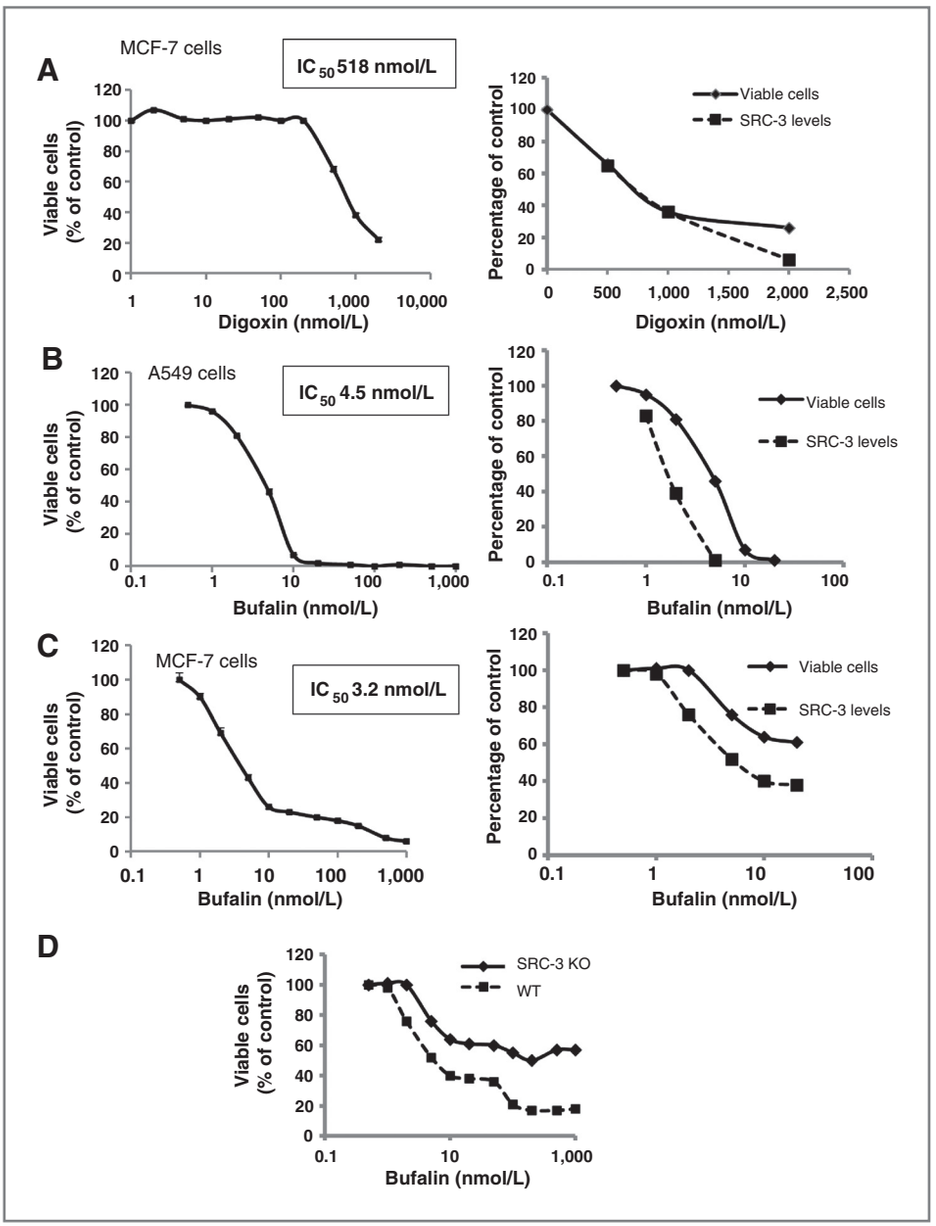

Figure 5. Digoxin and bufalin block breast and lung cancer cell proliferation. A, digoxin reduced MCF-7 cell viabilities in line with SRC-3 protein inhibition in a dose-dependent manner. Left, cells were treated with different doses (0, 1, 2, 5, 10, 20, 50, 100, 200, 500 nmol/L, 1, and 2 μmol/L) of digoxin for 24 hours, and then cell viability was determined by MTS assays. Collected data were normalized to the vehicle-treated control. Each data point represent the average of relative values derived from three wells of digoxin-treated cells at the indicated dose. Nonlinear regression was used to determine the point of 50% inhibition for cell viability (IC_{50}). Right, relative SRC-3 protein levels after digoxin (0.5, 1, and 2 μmol/L) treatment compared with the vehicle control (derived from Western blotting data in Fig. 4) are shown as the dashed line. B and C, bufalin reduced A549 (B) and MCF-7 (C) cell viabilities in accordance with SRC-3 protein degradation with increasing doses. Left, the dose responses for cell viabilities upon bufalin treatment (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 nmol/L) were analyzed as described above. Right, relative SRC-3 protein content after bufalin treatment (0, 1, 2, and 5 nmol/L) in A549 cells (derived from blotting shown in Fig. 4) and treatment (0, 1, 2, 5, and 10 nmol/L) in MCF-7 cells is shown as the dashed line. D, knockout of the SRC-3 protein attenuates the inhibitory effects of bufalin on cell viability. HeLa parental and SRC-3 knockout cells (SRC-3KO) were treated with bufalin at different doses (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 nmol/L) for 24 hours and cell viability was determined by MTS assay. HeLa parental cells are shown as the dashed line and the SRC-3KO cells are shown as the solid line.

compounds were then tested in the confirmatory screen in the same manner as the primary screen in quadruplicate and in a counter screen using cells transfected with an expression vector for a GAL4 DBD VP16 fusion protein instead of an expression vector for either GAL4 DBD SRC-3 or SRC-1 to exclude general inhibitors of transcription and/or luciferase activity.

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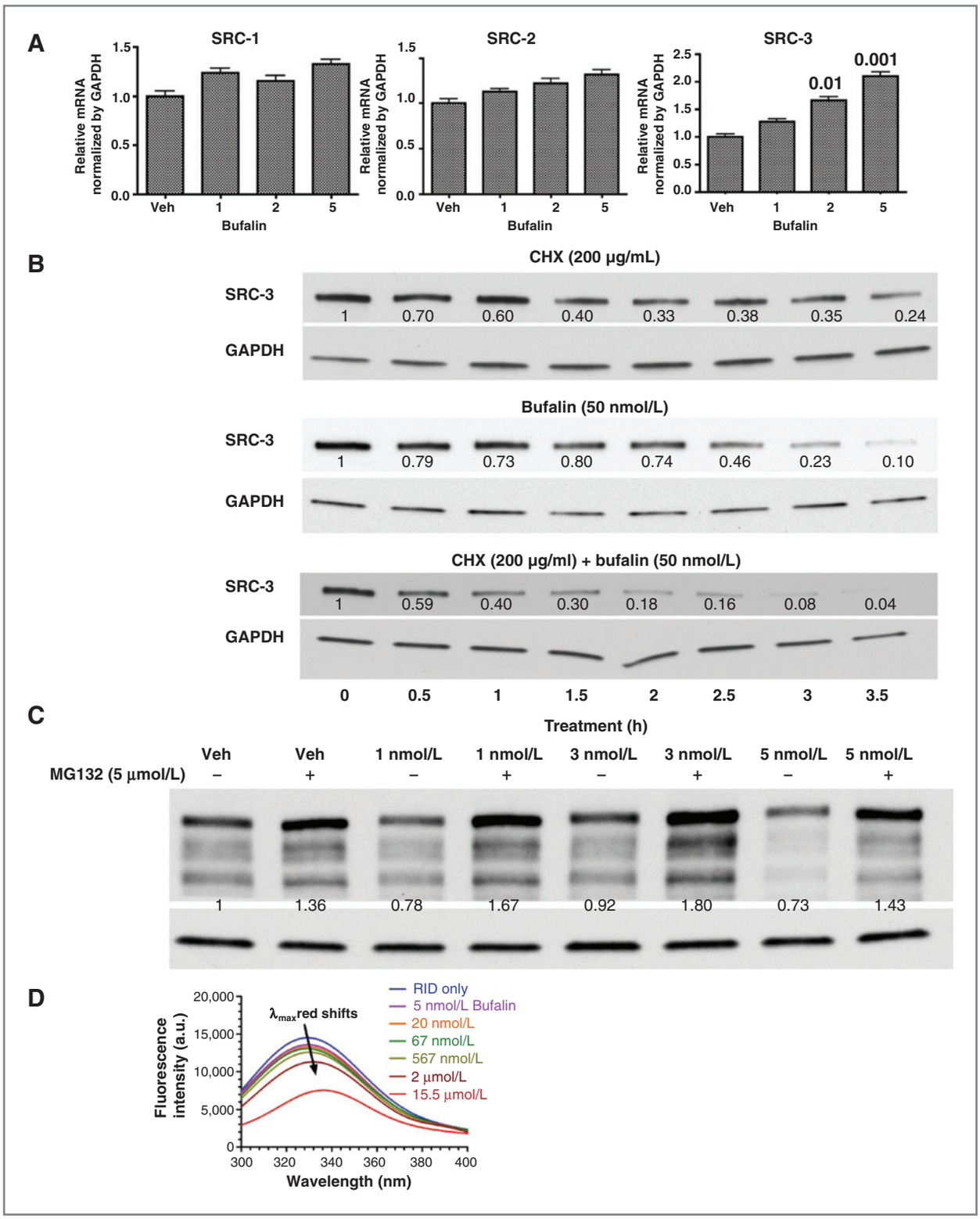

Figure 6. Bufalin promotes proteasome-mediated degradation of the SRC-3 protein. A, effects of bufalin on the mRNA levels of SRC-1, SRC-2, and SRC-3. MCF-7 cells were incubated with bufalin (0, 1, 2, and 5 nmol/L) for 24 hours and total RNA was extracted and analyzed by qPCR. Differences between the control and the treated samples were analyzed for significance using a Student *t* test. Only *P* values less than 0.05 are shown in the chart above its corresponding column. B, effects of bufalin on SRC-3 protein turnover rates. (*Continued on the following page.*)

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Structure–activity cluster analyses identify cardiac glycosides as a functional group of SRC-3 and SRC-1 SMIs

Active compounds retrieved from the primary screens were clustered according to the structural similarities in PubChem (data not shown). The biggest cluster contains 25 compounds, sharing a common steroid nucleus, the majority of which contain a lactone moiety characteristic of cardiac glycosides (35). Cardiac glycosides are known to inhibit the Na^+/K^+ ATPase in cardiac myocytes, leading to an increase in intracellular Ca^{2+} and stronger myocardial contraction (36). Interestingly, numerous epidemiologic studies revealed that patients taking cardiac glycosides have either better outcome or lower risk for various cancers, including breast cancers (37, 38), leukemia/lymphoma (39), and prostate cancer (40), although the mechanism has hitherto been unknown.

Evaluation of cardiac glycoside family members for their efficacy as SRC SMIs

Because a cluster of cardiac glycoside series was identified as active compounds in our high-throughput screens, we sought to evaluate a panel of cardiac glycosides to identify which were most potent as SRC SMIs. We examined SRC-3 protein concentrations in MCF-7 cells treated for 24 hours with cardiac glycosides, including ouabain, digitoxin, strophanthidin, cinobufagin, cinobufotalin, and resibufogenin. All tested compounds downregulated cellular SRC-3 protein levels, but at varying doses (Fig. 2). Ouabain and digitoxin seemed to be more effective than digoxin, while bufalin was found to be the most potent of all. In comparison, strophanthidin also reduced SRC-3 protein, but at a higher dose than digoxin. Bufalin was the most potent bufadienolide, followed (in order) by cinobufagin, cinobufotalin, and resibufogenin.

Inhibition of SRC-3 and SRC-1 by digoxin and bufalin

Next, we chose to investigate the effects of cardiac glycosides on the intrinsic transcriptional activities of SRC coactivators. HeLa cells were transiently transfected with a pGL5-LUC reporter and expression vectors for pBIND, pBIND-SRC-1, -SRC-2, or -SRC-3, followed by 24 hours of treatment with the plant-based cardiac glycoside digoxin or the toad cardiac glycoside bufalin. Digoxin reduced luciferase reporter activities in cells transfected with pBIND-SRC-1 and pBIND-SRC-3 (Fig. 3A). In contrast, reporter activities driven by the GAL4 DBD alone were only slightly affected (pBIND, inset to the right). Similarly, administration of bufalin led to a significant decrease in pBIND-SRC-1 and pBIND-SRC-3 activities in a dose-dependent manner, while only minimally affecting the activity of pBIND (GAL4 DBD alone) and influencing pBIND-SRC-2 less strongly (Fig. 3B). This result suggests that both digoxin and bufalin preferentially inhibit the intrinsic transcriptional activities of SRC-3 and SRC-1, while inhibiting SRC-2 to a lesser extent.

Effects of digoxin and bufalin on cellular protein concentrations of SRCs and other coactivators

Because the steady-state levels of coactivator proteins have been shown to correlate with their transcriptional activities and with cancer progression (33), we sought to examine the effects of digoxin and bufalin on SRC protein levels in MCF-7 breast and A549 lung cancer cells after 24 hours of incubation. As shown in Fig. 4A–C, SRC-3 and SRC-1 protein levels were significantly reduced by both digoxin and bufalin in a dose-dependent manner, suggesting that the loss in SRC-3 and SRC-1 intrinsic activities seen above is due to the reduced cellular concentration of either coactivator. In agreement with the finding that SRC-2 activities are less strongly affected, both digoxin and bufalin have more modest effects on the cellular level of the SRC-2 protein. We then investigated the steady-state level of CARM1 that has been shown to exist in a multiprotein coactivator complex along with SRC-3 (41). In contrast to the noticeable decrease in SRC-3 protein level, CARM1 protein level is not altered after treatment in A549 cells, but was reduced moderately in MCF-7 cells.

Digoxin and bufalin inhibit cancer cell proliferation

Next, we assessed the effects of digoxin and bufalin on cancer cell proliferation. First, MTS assays were performed on MCF-7 cells treated with digoxin at different doses for 24 hours. This revealed that digoxin can block MCF-7 cell growth with IC_{50} of about 500 nmol/L (Fig. 5A, left), in line with the dose of digoxin required to reduce SRC-3 protein levels in the cell (Fig. 5A, right). In contrast, the IC_{50} of bufalin in MCF-7 and A549 cells were below 5 nmol/L in either cell lines (Fig. 5B and C, left). Again, the dose of bufalin required to block cancer cell proliferation corresponds with the concentration required to cause downregulation of the SRC-3 protein (Fig. 5B and C, right). Differential dependence of distinct cell types toward each of the three SRCs could account for the differences in bufalin effect on cell viability and SRC-3 cellular protein concentration. Importantly, the concentrations of bufalin required to kill cancer cells tested here are less than the reported human maximum-tolerated dose (MTD) of 8.75 nmol/L (42), pointing to its potential clinical use as an anticancer agent. For this reason, we chose to focus on bufalin for further characterization as an SRC SMI.

Accumulating evidence has shown that targeting SRC-3 expression impairs cancer cell growth in multiple cancer types (8). Given the fact that the decreased cell viability induced by bufalin is accompanied by reduced SRC-3 protein levels, we sought to investigate the specific role of the SRC-3 protein in blocking cancer cell proliferation. To do this, we used a zinc finger nuclease (ZFN) to knockout both SRC-3 alleles in the HeLa cell line (SRC-3KO cells). It was difficult to obtain viable SRC-3KO cells initially; however, we noted that after 2 months,

(*Continued.*) MCF-7 cells were treated with cycloheximide (CHX; 200 μg/mL) and bufalin (50 nmol/L), individually or simultaneously, and then harvested at 0, 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 hours thereafter and visualized by Western blot analysis. C, the proteasome inhibitor MG132 prevents SRC-3 protein downregulation induced by bufalin. MCF-7 were changed to medium supplemented with 0.5% stripped FCS, penicillin, and streptomycin (100 U/mL) overnight, and then incubated with bufalin (0, 1, 3, and 5 nmol/L) in the absence or presence of 5 μmol/L MG132 for 24 hours. Cell extracts were collected and analyzed by Western blotting using antibodies against SRC-3 and GAPDH. D, bufalin directly binds to the RID of SRC-3. Intrinsic tryptophan fluorescence emission spectra of SRC-3 RID ($\lambda_{ex} = 278$ nm) were quenched and redshifted with increasing concentrations of bufalin.

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they proliferated at a rate comparable with wild-type (WT) cells, likely after adapting genetically to the loss of SRC-3. As shown in Fig. 5D, SRC-3 protein is abolished in these SRC-3KO knockout cells. Compared with parental SRC-$3^{+/+}$ cells, the response of SRC-3KO cells to bufalin administration is blunted. This finding supports the idea that SRC-3 protein is involved in mediating the cell response to bufalin treatment. However, the remaining response of SRC-3KO cells to bufalin, including the lack of an increase in the effective concentration required for these cells to respond to treatment, is likely due to the SRC-1 and SRC-2 that continues to be expressed in these cells and that also responds to bufalin at a similar dose as SRC-3, in addition to any unknown off-target actions of the compound.

Bufalin promotes proteasome-mediated degradation of SRC-3 and binds directly to the coactivator

To gain insights into the mechanism of bufalin-mediated SRC-3 protein downregulation, we assessed whether 24 hours of bufalin treatment affected the production of mRNAs for each SRC family member in MCF-7 cells. Quantitative PCR (qPCR) revealed that mRNA levels for SRC-1 and SRC-2 were not significantly altered, whereas the mRNA levels for SRC-3 were actually increased upon bufalin incubation (Fig. 6A). This result suggested that bufalin reduces SRC-3 and SRC-1 protein levels posttranscriptionally.

To further investigate the underlying mechanism responsible for bufalin-mediated SRC-3 protein downregulation, we compared the turnover rates of the SRC-3 protein in MCF-7 cells between vehicle and bufalin treatment in the presence of the protein synthesis inhibitor cycloheximide. Cells were treated with cycloheximide and bufalin, individually or simultaneously, and then harvested at the indicated time points for Western blot analysis. In line with a previous report (43), SRC-3 decays with a 4-hour half-life (Fig. 6B). The addition of bufalin accelerated the rate of SRC-3 turnover, indicating that bufalin promotes degradation of the SRC-3 protein.

Our laboratory has previously shown that all SRC coactivators are targets of the proteasome, as evidenced by the observation that treatment with the proteasome inhibitor MG132 increases their protein levels (30). When we examined the effects of concomitant treatment with MG132 and bufalin ranging from 1 to 5 nmol/L on SRC-3 protein levels in MCF-7 cells (Fig. 6C), MG132 treatment alone was able to strongly elevate the SRC-3 protein levels, consistent with that previously reported. The reduction in SRC-3 protein level induced by bufalin treatment was blocked by MG132, leading to protein levels up to a point comparable with cells treated with MG132 alone.

Next, we sought to determine whether bufalin promoted SRC-3 protein degradation through physical interaction with the SRC-3 protein. Through fluorescence shift analysis of SRC-3 protein fragments, bufalin was found to quench the intrinsic fluorescence and shift the emission maximum of the RID of SRC-3 (Fig. 6D), indicating that bufalin binds directly to the RID of SRC-3. In contrast, there are no changes in the intrinsic fluorescence observed for the CID or the bHLH domain of SRC-3 upon the addition of bufalin (data not shown), which mitigates the possibility that the fluorescence changes seen for the RID fragment are due to nonspecific interactions with bufalin. Because of the high affinity of bufalin binding to the SRC-3 RID, it is difficult to precisely determine its affinity; however, binding was detected even with 5 nmol/L of bufalin, consistent with its potency in cell culture experiments.

Bufalin sensitizes cancer cells to other targeted therapeutics

Accumulating evidence shows that SRC coactivators sit at a nexus linking diverse growth signaling cascades (see Introduction). This role for SRCs supports the notion that an SRC SMI such as bufalin should break cross-talk between different growth factor pathways, attenuating their ability to signal to downstream pathway components responsible for enacting cellular transcriptional programs that drive cell proliferation, invasion, and metastasis. SRC-3 has been shown to activate PI3K/AKT/mTOR signaling (44), and we wanted to ask whether the inhibitory effects of an AKT inhibitor on cell viability could be enhanced by cotreatment with bufalin. To test this, we treated A549 cells with a low dose of bufalin in combination with the AKT inhibitor MK-2206. As shown in Fig. 7A, combined treatment with bufalin and MK-2206 exhibited a markedly higher ability to block A549 cell proliferation than either with agent alone.

A bufalin nanoparticle formation can block tumor cell growth in a xenograft model

Bufalin is an excellent candidate for incorporation into a nanoparticle-based delivery system because it can specifically kill cancer cells (IC_{50}, 3–5 nmol/L) without observable toxicity in nontransformed TM4 Sertoli cells at doses up to 10 μmol/L (Fig. 7B) or primary hepatocytes even at doses as high as 300 nmol/L (data not shown), but it possesses significant cardiotoxicity—a fact we substantiated in our own animal studies with free bufalin (data not shown), and it has a short *in vivo* half-life. With "free bufalin," we observed a response in metastatic tumor lesions using an *in vivo* model described below, but we were unable to achieve a satisfactory response in primary tumors, possibly due to dose-limiting toxicities or due to inadequate drug penetration into larger tumors (data not shown). Already, a wheat germ agglutinin–grafted lipid bufalin nanoparticle has been developed to allow for its oral delivery and improve its stability (45). Another formulation consists of a methoxy PEG, PLGA, poly-L-lysine, and cyclic arginine-glycine-aspartic acid carrier loaded with bufalin that has been shown to possess a favorable biodistribution profile and has antitumor activity *in vivo* (45). Here, we chose to test a similar and established PEG-PLGA–based nanoparticle delivery system (see Materials and Methods; ref. 46) that has already been successfully used to produce doxorubicin PEG–PLGA nanoparticles that have been designed to avoid distribution to cardiac tissue.

Fox Chase SCID Beige mice were injected with 750,000 MDA-MB-231–derived LM3.3 cells into the second mammary gland (cleared) with two sites per mice. Six days after tumor cell injection, mice were separated into three groups and given the following treatments: (i) PBS vehicle control–treated ($n = 5$); (ii) bufalin nanoparticle (1.5 mg/kg)–treated ($n = 10$); and (iii)

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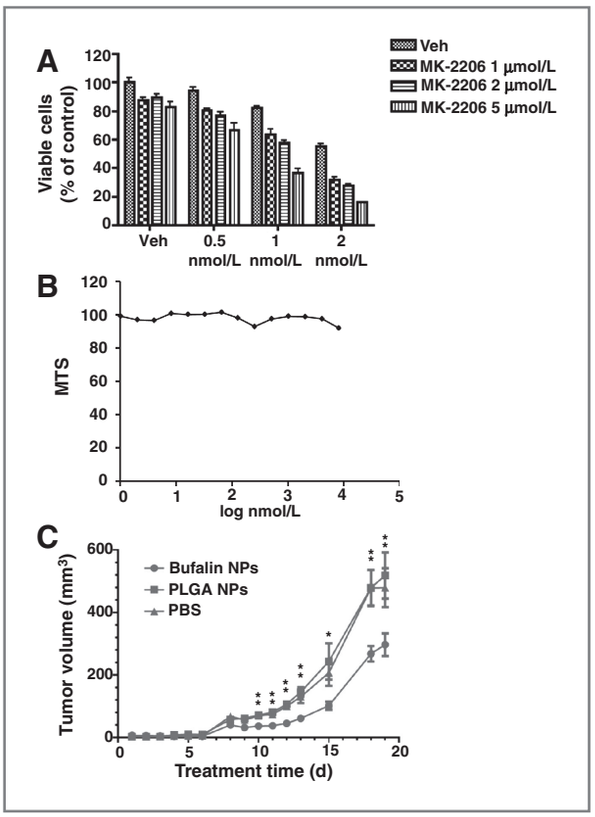

Figure 7. A, bufalin sensitizes cancer cells to other targeted chemotherapeutic agents and blocks tumor growth *in vivo*. When combined with 1 or 2 nmol/L bufalin, cotreatment with the Akt inhibitor MK-2206 significantly reduced lung cancer A549 cell viability (1, 2, and 5 μmol/L). Cell viability was determined by MTS assays, with data normalized to the vehicle treatment control, set to 100%. Each data point represents the average from three data points. B, nontransformed TM4 Sertoli cells were treated with bufalin at the indicated concentrations. Seventy-two hours after treatment, cell viability was determined via MTS assay. C, bufalin nanoparticle (NP) inhibition of tumor growth *in vivo*. SCID mice were injected with 750,000 LM3.3 cells into the second mammary gland (cleared) with two sites per mice. Six days after tumor cell injection, mice were treated with PBS vehicle, bufalin nanoparticles (1.5 mg/kg), or with blank nanoparticles that were not loaded with bufalin three times per week (*, $P < 0.05$; **, $P < 0.01$ for comparisons between blank nanoparticles and bufalin-loaded nanoparticles).

blank nanoparticle-treated ($n = 5$). Mice were treated three times per week via intravenous injection. After this, primary tumor volume was measured to assess the ability of the bufalin nanoparticle to inhibit tumor growth (Fig. 7C). Aggressive tumor growth was observed in the PBS- and blank nanoparticle-treated control mice, whereas the bufalin nanoparticle-treated mice showed a significant inhibition in the rate of tumor growth 4 days after initial treatment that was sustained throughout the experiment.

Discussion

The SRC family has been widely implicated in carcinogenesis, providing a strong impetus to develop SRC SMIs as novel and effective therapeutic agents. Using a luminescence-based assay to assess coactivator intrinsic transcriptional activities, we conducted a high-throughput screen of an MLPCN compound library (34) to identify SRC-3 and SRC-1 SMI hits. Because of the large size of this library, we were able to gain insight into the comparative activities of many structurally related compounds, revealing cardiac glycosides as the largest class of compounds with SRC SMI activities. Our data demonstrate that both digoxin and bufalin selectively reduced intrinsic activities of SRC-3 and SRC-1, consistent with that observed in the primary screen. Marked differences in the potencies of cardiac glycosides were observed, with most inhibiting SRCs at concentrations higher than their MTD. However, bufalin was found to have better potency and inhibited SRCs at concentrations below its MTD of 8.75 nmol/L, leading to our focus on it as a potentially clinically useful SRC SMI.

For centuries, cardiac glycosides have been used to treat patients with edematous states, irregular heartbeats, or chronic heart failure, and epidemiologic evidence has shown that patients who take cardiac glycosides are at lower risk for various cancers, pointing to their potential as cancer therapeutic agents (47). Interestingly, cardiac glycosides have also been shown to inhibit the androgen receptor, but were found not to bind to the receptor itself (48).

Despite their anticancer properties, the effectiveness of cardiac glycosides is limited by their narrow therapeutic window. For example, the nontoxic plasma concentration of digoxin for cardiac disease patients is 2.6 nmol/L or less (49). In our study, the concentration of digoxin required to inhibit SRC-3/SRC-1 is greater than 200 nmol/L. In contrast, we show here that bufalin is effective at a low nanomolar range (~3–5 nmol/L), which is within the concentration range observed in patient plasma in which no cardiac toxicity was observed (~9 nmol/L; ref. 42). Given the enhanced effectiveness when combined with the AKT inhibitor MK-2206, bufalin's ability to block tumor growth might be achievable at even lower doses. Another approach we pursued to avoid the dose-limiting toxicity associated with bufalin toward the heart was to deliver bufalin in a PEG–PLGA delivery particle, in which we were able to effectively block breast tumor growth *in vivo*. Bufalin has already been tested in several small clinical trials (42) and the data presented here that characterize it as an SRC SMI provide new insight into its mechanism of action. Results presented here demonstrating that SRC-3 and SRC-1 are targets of bufalin promise to more rationally guide its future use as a novel therapeutic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Wang, D.M. Lonard, D.-C. Chow, J. Wang, X. Song, P. Hodder, B.W. O'Malley

Development of methodology: Y. Wang, D.M. Lonard, Y. Yu, D.-C. Chow, J. Wang, X. Song, P. Hodder, L. Liao, J. Xu, B.W. O'Malley

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Wang, D.M. Lonard, Y. Yu, D.-C. Chow, A.J. Matzuk, X. Song, F. Madoux, P. Hodder, P. Chase, S. Zhou, L. Liao, J. Xu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, D.M. Lonard, Y. Yu, D.-C. Chow, T.G.

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Palzkill, R. Qi, A.J. Matzuk, X. Song, F. Madoux, P. Hodder, P. Chase, P.R. Griffin, L. Liao, B.W. O'Malley

Writing, review, and/or revision of the manuscript: Y. Wang, D.M. Lonard, D.-C. Chow, J. Wang, F. Madoux, P. Hodder, B.W. O'Malley

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.M. Lonard, D.-C. Chow, F. Madoux, P. Hodder, B.W. O'Malley

Study supervision: D.M. Lonard, T.G. Palzkill, P. Hodder, P.R. Griffin, B.W. O'Malley

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References

1. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270:1354–7.
2. Hong H, Kohli K, Garabedian MJ, Stallcup MR. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol 1997;17:2735–44.
3. Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 1996;15:3667–75.
4. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.
5. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997;90:569–80.
6. Li H, Gomes PJ, Chen JD. RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc Natl Acad Sci U S A 1997;94:8479–84.
7. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, et al. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 1997;387:677–84.
8. Lonard DM, Lanz RB, O'Malley BW. Nuclear receptor coregulators and human disease. Endocr Rev 2007;28:575–87.
9. Mussi P, Yu C, O'Malley BW, Xu J. Stimulation of steroid receptor coactivator-3 (SRC-3) gene overexpression by a positive regulatory loop of E2F1 and SRC-3. Mol Endocrinol 2006;20:3105–19.
10. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, et al. Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol 1999;19:6367–78.
11. Louie MC, Zou JX, Rabinovich A, Chen HW. ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. Mol Cell Biol 2004;24:5157–71.
12. Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 2004;6:263–74.
13. Fereshteh MP, Tilli MT, Kim SE, Xu J, O'Malley BW, Wellstein A, et al. The nuclear receptor coactivator amplified in breast cancer-1 is required for Neu (ErbB2/HER2) activation, signaling, and mammary tumorigenesis in mice. Cancer Res 2008;68:3697–706.
14. Kuang SQ, Liao L, Wang S, Medina D, O'Malley BW, Xu J. Mice lacking the amplified in breast cancer 1/steroid receptor coactivator-3 are resistant to chemical carcinogen-induced mammary tumorigenesis. Cancer Res 2005;65:7993–8002.
15. Kirkegaard T, McGlynn LM, Campbell FM, Muller S, Tovey SM, Dunne B, et al. Amplified in breast cancer 1 in human epidermal growth factor receptor-positive tumors of tamoxifen-treated breast cancer patients. Clin Cancer Res 2007;13:1405–11.
16. Lahusen T, Fereshteh M, Oh A, Wellstein A, Riegel AT. Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1. Cancer Res 2007;67:7256–65.
17. Bautista S, Valles H, Walker RL, Anzick S, Zeillinger R, Meltzer P, et al. In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. Clin Cancer Res 1998;4:2925–9.
18. Kershah SM, Desouki MM, Koterba KL, Rowan BG. Expression of estrogen receptor coregulators in normal and malignant human endometrium. Gynecol Oncol 2004;92:304–13.
19. Gnanapragasam VJ, Leung HY, Pulimood AS, Neal DE, Robson CN. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. Br J Cancer 2001;85:1928–36.
20. Maki HE, Waltering KK, Wallen MJ, Martikainen PM, Tammela TL, van Weerden WM, et al. Screening of genetic and expression alterations of SRC1 gene in prostate cancer. Prostate. 2006;66:1391–8.
21. Wang Y, Wu MC, Sham JS, Zhang W, Wu WQ, Guan XY. Prognostic significance of c-myc and AIB1 amplification in hepatocellular carcinoma. A broad survey using high-throughput tissue microarray. Cancer 2002;95:2346–52.
22. Henke RT, Haddad BR, Kim SE, Rone JD, Mani A, Jessup JM, et al. Overexpression of the nuclear receptor coactivator AIB1 (SRC-3) during progression of pancreatic adenocarcinoma. Clin Cancer Res 2004;10:6134–42.
23. Xie D, Sham JS, Zeng WF, Lin HL, Bi J, Che LH, et al. Correlation of AIB1 overexpression with advanced clinical stage of human colorectal carcinoma. Hum Pathol 2005;36:777–83.
24. Cai D, Shames DS, Raso MG, Xie Y, Kim YH, Pollack JR, et al. Steroid receptor coactivator-3 expression in lung cancer and its role in the regulation of cancer cell survival and proliferation. Cancer Res 2010;70:6477–85.
25. Wang S, Yuan Y, Liao L, Kuang SQ, Tien JC, O'Malley BW, et al. Disruption of the SRC-1 gene in mice suppresses breast cancer metastasis without affecting primary tumor formation. Proc Natl Acad Sci U S A 2009;106:151–6.
26. Fleming FJ, Myers E, Kelly G, Crotty TB, McDermott EW, O'Higgins NJ, et al. Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer; a predictive role for SRC-1. J Clin Pathol 2004;57:1069–74.
27. Myers E, Fleming FJ, Crotty TB, Kelly G, McDermott EW, O'Higgins NJ, et al. Inverse relationship between ER-beta and SRC-1 predicts outcome in endocrine-resistant breast cancer. Br J Cancer 2004;91:1687–93.
28. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, O'Malley BW. Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. Mol Endocrinol 2011;25:2041–53.
29. NIH Roadmap Molecular Libraries Initiative [Internet]. Bethesda (MD): The National Institutes of Health. c2007 - [cited 2013 Dec 23]. Available from: https://mli.nih.gov/mli/compound-repository/mlsmr-compounds/.
30. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell 2000;5:939–48.
31. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012:9:671–5.
32. PubChem Bioassay [Internet]. Bethesda (MD): The National Center for Biotechnology Information, U.S. National Library of Medicine. c2007 - [cited 2013 Dec 23]. Available from: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=588354.

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33. Wu RC, Feng Q, Lonard DM, O'Malley BW. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. Cell 2007;129:1125–40.
34. Roy A, McDonald PR, Sittampalam S, Chaguturu R. Open access high throughput drug discovery in the public domain: a Mount Everest in the making. Curr Pharm Biotechnol 2010;11:764–78.
35. Schatzmann HJ. The role of Na^+ and K^+ in the ouabain-inhibition of the Na^+ $+K^+$-activated membrane adenosine triphosphatase. Biochim Biophys Acta 1965;94:89–96.
36. Lip GY, Watson RD, Singh SP. ABC of atrial fibrillation. Drugs for atrial fibrillation. BMJ 1995;311:1631–4.
37. Stenkvist B, Bengtsson E, Eriksson O, Holmquist J, Nordin B, Westman-Naeser S. Cardiac glycosides and breast cancer. Lancet 1979;1:563.
38. Stenkvist B, Bengtsson E, Dahlqvist B, Eriksson O, Jarkrans T, Nordin B. Cardiac glycosides and breast cancer, revisited. N Engl J Med 1982;306:484.
39. Haux J, Klepp O, Spigset O, Tretli S. Digitoxin medication and cancer; case control and internal dose-response studies. BMC Cancer 2001;1:11.
40. Platz EA, Yegnasubramanian S, Liu JO, Chong CR, Shim JS, Kenfield SA, et al. A novel two-stage, transdisciplinary study identifies digoxin as a possible drug for prostate cancer treatment. Cancer Discov 2011;1:68–77.
41. Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, et al. Regulation of transcription by a protein methyltransferase. Science 1999;284:2174–7.
42. Meng Z, Yang P, Shen Y, Bei W, Zhang Y, Ge Y, et al. Pilot study of huachansu in patients with hepatocellular carcinoma, non-small-cell lung cancer, or pancreatic cancer. Cancer 2009;115:5309–18.
43. Lonard DM, Tsai SY, O'Malley BW. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. Mol Cell Biol 2004;24:14–24.
44. Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ. Role of the steroid receptor coactivator SRC-3 in cell growth. Mol Cell Biol 2003;23:7742–55.
45. Yin P, Wang Y, Qiu Y, Hou L, Liu X, Qin J, et al. Bufalin-loaded mPEG–PLGA–PLL–cRGD nanoparticles: preparation, cellular uptake, tissue distribution, and anticancer activity. Int J Nanomedicine 2012;7:3961–9.
46. Yoo HS, Park TG. Biodegradable polymeric micelles composed of doxorubicin conjugated PLGA–PEG block copolymer. J Control Release 2001;70:63–70.
47. Cerella C, Dicato M, Diederich M. Assembling the puzzle of anti-cancer mechanisms triggered by cardiac glycosides. Mitochondrion 2012;13:225–34.
48. Li H, Zhou H, Wang D, Qiu J, Zhou Y, Li X, et al. Versatile pathway-centric approach based on high-throughput sequencing to anticancer drug discovery. Proc Natl Acad Sci U S A 2012;109:4609–14.
49. Smith TW, Butler VP Jr, Haber E. Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. N Engl J Med 1969;281:1212–6.
50. NCBI PubChem Database [Internet]. Bethesda (MD): The National Center for Biotechnology Information, U.S. National Library of Medicine. c2007 [cited 2013 Dec 23]. Available from: https://pubchem.ncbi.nlm.nih.gov/.

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Nuclear receptor modulation – Role of coregulators in selective estrogen receptor modulator (SERM) actions

Qin Feng, Bert W. O'Malley *

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

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ABSTRACT

Selective estrogen receptor modulators (SERMs) are a class of small-molecule chemical compounds that bind to estrogen receptor (ER) ligand binding domain (LBD) with high affinity and selectively modulate ER transcriptional activity in a cell- and tissue-dependent manner. The prototype of SERMs is tamoxifen, which has agonist activity in bone, but has antagonist activity in breast. Tamoxifen can reduce the risk of breast cancer and, at same time, prevent osteoporosis in postmenopausal women. Tamoxifen is widely prescribed for treatment and prevention of breast cancer. Mechanistically the activity of SERMs is determined by the selective recruitment of coactivators and corepressors in different cell types and tissues. Therefore, understanding the coregulator function is the key to understanding the tissue selective activity of SERMs.

1. Introduction

The pleiotropic effects of estrogens are mediated by their two cognate nuclear receptors, estrogen receptor alpha and beta (ERα and ERβ), which are members of the nuclear hormone receptor superfamily [1]. Estrogens are essential for the maintenance of normal functions of the female reproductive tissues and non-reproductive tissues including metabolic homeostasis, skeletal homeostasis, the cardiovascular system, and central nervous system (CNS) [2,3]. Diminished estrogen levels in postmenopausal women are associated with dysfunctions in those tissues. Hormone replacement therapy (HRT; estrogen plus progestin) is effective in relieving the symptoms associated with menopause but is associated with an increased incidence of breast cancer. Replacement with pure estrogen is associated with increased risk of uterine cancers [4,5]. Selective estrogen receptor modulators (SERMs) can selectively activate or inhibit ER transcriptional activity in different tissues, and therefore retain beneficial effects while reducing harmful effects [6]. SERMs have been widely used for treatment of ER-positive breast cancer which occurs in 70% of breast cancer cases. The most commonly used SERMs in clinic is tamoxifen, and it has been used clinically for more than 30 years as a front-line treatment for the ERα-positive breast tumors at all stages [7]. Other second generation SERMs that have decreased stimulatory effects on the uterus are now available.

2. Structure and function of estrogen receptors

Estrogen receptors ERα and ERβ contain three major functional domains including an amino-terminal Activation Function-1 (AF-1) domain, a central DNA-binding domain (DBD), and a carboxyl-terminal ligand binding domain (LBD) (Fig. 1A). The variable NH_2-terminal AF-1 domains of ERs are not conserved among the nuclear receptor superfamily. The AF-1 domain is intrinsically unstructured in solution and forms secondary structure when engaged in interaction with coregulators. The transcriptional activity of AF-1 is controlled by phosphorylation through the MAPK pathway [8,9] and is hormone-independent. The centrally located DBD is the most conserved region and is a signature domain of nuclear receptors. The DBD is composed of two C4-type zinc fingers and recognizes specific DNA sequences in enhancer or promoter regions of target genes, known as hormone response elements (HREs). The carboxyl-terminal LBD contains 12 alpha helices (H1–H12) which form alpha helical sandwich conformation [10]. The hydrophobic ligand binding cavity is within the interior of the LBD and binds estrogen with high specificity and affinity. Hormone binding subsequently induces a conformational change and creates a novel interacting surface for coregulators. The primary sequence of LBDs is moderately conserved among nuclear receptors.

ERα mainly functions as a transcription factor in the nucleus. Hormone-bound ER protein binds to estrogen response elements (EREs) located on enhancer/promoter regions of target genes, and directly regulates gene expression in response to estrogens. ERα

* Corresponding author. Tel.: +01 713 798 6205; fax: +01 713 798 5599.
E-mail address: berto@bcm.edu (B.W. O'Malley).

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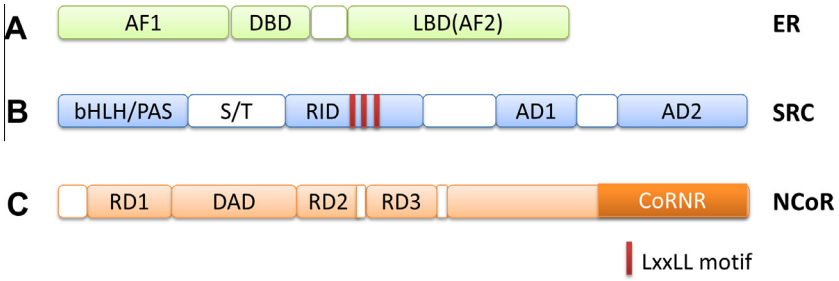

Fig. 1. Domain structure of ER, SRC coactivators and NCoR corepressors. (A) ER is composed of NH2-terminal AF1, DBD (DNA binding domain), and carboxyl-terminal LBD (Ligand binding domain). There is a hinge region between DBD and LBD. (B) SRC coactivators contain several major domains, including bHLH/PAS, RID that contains three LxxLL motifs, AD1 that interacts with p300/CBP, and AD2 that interacts with CARM1. (C) NCoR coreporessors, NCoR1 and NCoR2 (SMRT), contain several repression domains including RD1, RD2, RD3 and DAD (deacetylase activating domain) that interact with HDACs, and CoRNR (nuclear receptor interacting domain in corepressors) domain that interacts with nuclear receptor.

protein itself does not possess intrinsic enzymatic activity. Instead ERα recruits coregulators that contain diverse enzymatic activities, including histone acetyltransferase (HAT), histone methyltransferase (HMT), kinase, etc. These coregulators have no DBD and cannot bind directly to genomic DNA. They are targeted to specific genomic sites by interactions with DNA-bound ER protein. Once enriched at the specific genomic regions, the coregulators can covalently modify histones and DNA, and subsequently alter chromatin structure to facilitate or suppress the transcription of target genes. Thus, the transcriptional activity of ER is essentially carried out by coregulators. More than 450 coregulators have been reported for nuclear hormone receptors in the literature, and many of them contain a variety of different enzymatic activities.

3. The SRC family of transcriptional coactivators are "primary" ER coactivators

The SRC/p160 family of coactivators are the best characterized coactivators for steroid hormone receptors including ER. This family consists of three members. Steroid receptor coactivator 1 (SRC-1) is the first bona fide nuclear receptor coactivator, cloned in 1995 as an interacting partner for the progesterone receptor LBD through a yeast two-hybrid screening [11]. The subsequent identification and characterization of SRC-2 (GRIP1, TIF2) and SRC-3 (p/CIP, RAC3, ACTR, AIB1, and TRAM-1) has established the SRC/p160 family of coactivators [12]. Although SRCs are named as coactivators for steroid hormone receptors, it is clear that they also function as coactivators for non-steroid groups of nuclear receptors, such as thyroid hormone receptors, retinoid receptors, vitamin D3 receptor, and many non-nuclear receptor transcription factors as well, such as E2F1 [13], Ets-2 [14] and NF-κB [15].

The three SRC family members are highly homologous and share an overall similarity of 50–55% in their primary sequences [16]. SRC coactivators contain several functional domains, including a NH_2-terminal basic helix-loop-helix-Per/Ah receptor nuclear translocation/Sim (bHLH/PAS) domain, a receptor interacting domain (RID), and carboxyl-terminal Activation Domains 1 (AD1) and 2 (AD2) (Fig. 1B). The RID contains three amphipathic leu-x-x-leu-leu (LxxLL) motifs [17] and is responsible for direct interaction with agonist-bound receptor LBD [18]. Upon estrogen binding, the carboxyl-terminal alpha helix 12 of ER folds back toward the ER LBD and, together with helix 3 and 5, forms a hydrophobic cleft, which interacts with the hydrophobic surface of LxxLL motifs of SRCs. Because they are directly recruited by hormone-bound ER, SRC coactivators are considered as primary coactivators. Subsequently SRCs serve as bridging molecules to bring in secondary coactivators through AD1 and AD2, including p300/CBP [19] and CARM1 [20]. p300 and CBP are potent histone acetyltransferases, whereas CARM1 is a histone methyltransferase. The AD1 of SRCs physically associates with p300/CBP and shows potent transcriptional activity in reporter gene assay when tethered to a heterologous DNA binding domain such as GAL4 DBD [21]. AD2 binds to CARM1 and shows relatively weaker transcriptional activity when tethered to GAL4 DBD [21]. Interestingly SRC-1 and SRC-3 also possess intrinsic acetyltransferase activity toward histones [19,22]. However their HAT activity is weak, suggesting that their cognate substrates might not be histones.

In addition to conventional reporter gene assays, recruitment of SRC coactivators by ER to its target genes is strongly supported by state-of-the-art genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis. In MCF7 breast cancer cells, mapping of the chromatin binding sites of the three members of SRCs revealed that the genomic recruitment of SRC proteins clearly followed ERα ligand stimulation, indicating that SRC recruitment by ER indeed occurs in real time in cells [23]. However the targeting genes regulated by SRC-3 are not shared with the other two SRC family members. This is in agreement with other studies showing that each SRC protein regulates a unique set of genes in MCF7 cells, suggesting that they play non-redundant roles in breast cancer development [24].

There also is ample biochemical evidence showing that SRC coactivators are bona fide coactivators for ER. In immunoprecipitation-mass spectrometry analyses, purification of E2-bound ER-associated protein complexes revealed that all there SRC members interact with ER in a hormone-dependent manner [25]. Similarly, purification of SRC-3-associated protein complexes confirms that CBP/p300 form a complex with SRC-3 [25]. In chromatin-based *in vitro* transcription assays, SRCs and CBP/p300 significantly enhance ER-mediated transcription [26]. Our recent Cryo-EM study reveals that two SRC-3 molecules simultaneously interact with an ERα dimer on DNA and form an asymmetric protein surface, providing a structural basis for further recruitment of a large number of secondary coactivators (unpublished).

The *in vivo* biological roles of SRC coactivators in female reproductive functions in animal have been well documented. For instance, SRC-1 null mice show partial resistance to steroid hormones [27]. SRC-2 is essential for progesterone-dependent uterine function and mammary gland morphogenesis in mouse models [28]. SRC-3 is required for female reproductive function and mammary gland development [29]. Finally it is worthy to note that SRC-3/AIB1 is a bona fide oncogene in breast cancer. The SRC-3 gene is amplified in ~10% of breast tumor samples [30] and SRC-3 mRNA is overexpressed in the majority of primary breast cancers. Forced overexpression of SRC-3 in mammary gland caused mammary tumor development [31]. Collectively, these molecular, cellular, biochemical, and animal data have unequivocally established that SRC proteins are bona fide coactivators for nuclear receptors and are key components of the estrogen/ER signaling pathway.

4. The NCoR1/SMRT corepressor family

In contrast to coactivators, there are a group of transcriptional factors, termed corepressors, which oppose the action of coactivators in nuclear receptor-mediated transcriptional regulation; the existence of coactivators and corepressors suggests a yin-yang relationship for regulation of gene transcription. NCoR1 (nuclear receptor corepressor (1) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) are prototypical nuclear receptor corepressors. NCoR1 and SMRT were initially cloned as hormone-independent TR or RXR interacting proteins in yeast two-hybrid screening [32,33]. NCoR1 and SMRT interact with nuclear receptors in the absence of hormone and their interaction is disrupted by agonist binding. NCoR1 and SMRT contain a receptor interacting domain (RID) and repression domains (RDs) (Fig. 1C). The RID contains a L/I-x-x-I/V-I motif (CORNR motif) to interact with nuclear receptors [34–36], reminiscent of the L-x-x-L-L motif (also known as NR box) of coactivators. Importantly NCoR1 and SMRT do not have intrinsic enzymatic activity. Instead NCoR1 and SMRT function as scaffold proteins, and they recruit histone deacetylases including HDAC3 through several repression domains (RD1, RD2 and RD3) [37,38]. In the absence of hormone, nuclear receptors TR, RAR and RXR bind to enhancers of target genes, and recruit NCoR1 and/or SMRT and associated HDACs to remove histone acetylation marks that suppress gene transcription. When receptors bind an agonistic ligand, the corepressors NCoR1/SMRT and their associated HDACs are dissociated, and SRC coactivators in turn are loaded to the agonist-bound receptors to stimulate gene transcription [39].

It is important to note that although NCoR1 and SMRT were initially identified as corepressors for non-steroid nuclear receptors, later studies have clearly established that NCoR1/SMRT can also function as corepressors for steroid receptors and suppress their target gene transcription [40–43]. For example, NCoR1 is recruited to the pS2 gene promoter by ER in the absence of hormone, and tamoxifen treatment enhances NCoR recruitment [44]. NCoR1 and SMRT can also function as corepressors for many non-nuclear receptor transcriptional factors [45].

5. Determination of SERM activity by coactivators and corepressors

The unique feature of a SERM is its cell- and tissue-selective activity. The first implication that relative expression of coactivator and corepressors in cells contribute to SERM function is based on transient transfection luciferase reporter assays. In this study, overexpression of SRC-1 enhanced 4-hydroxytamoxifen (4HT)-stimulated ER activity, whereas overexpression of SMRT strongly suppressed 4HT-stimulated ER activity [40]. When SRC-1 and SMRT were co-overexpressed, SMRT blocked the SRC-1 coactivation of 4HT-depependent ER activity [40]. For the first time, these results suggested that the relative expression of SRC-1 and SMRT can modulate the 4HT-dependent ER activity.

This observation is further supported by a study showing that tamoxifen is antiestrogenic in breast cells but estrogenic in endometrial cells. It was found that a high expression level of SRC-1 in endometrial carcinoma cell lines is responsible for induction of c-Myc and IGF-1 by tamoxifen-stimulated ER. Growth stimulatory effects of tamoxifen in uterine cells were abolished by depletion of SRC-1 by siRNA, indicating the SRC-1 levels are critical for agonist activity of tamoxifen in the endometrium [46].

In an *in vitro* model, up-regulated SRC-3 coactivator expression is associated with acquired tamoxifen resistance in MCF-7 cells cultured in tamoxifen-containing media [47]. Importantly in breast cancer patients, high SRC-3 level also correlates with tamoxifen resistance [48]. In cultured breast cancer cells, overexpression of SRC-3 and Her2 is able to convert tamoxifen from an antagonist into an ER agonist, resulting in de novo resistance [49]. Under this condition, tamoxifen treatment causes recruitment of coactivator complexes (SRC-3 and p300/CBP) to, and dissociation of corepressor complexes (NCoR/HDAC3) from, the ER-regulated pS2 gene promoter. In agreement with this, depletion of SRC-3 expression by siRNA restored the inhibitory effect of tamoxifen on cell proliferation in breast cancer BT474 cells [50]. PKA-induced tamoxifen resistance is associated with increased recruitment of SRC-1 by phosphorylation of S305 [51].

On the other hand, in breast cancer cells, corepressor NCoR1 and SMRT are required for the antagonistic effects of tamoxifen. They prevent tamoxifen from stimulating proliferation through suppression of a subset of ERα target genes involved in cell proliferation. Silencing of NCoR1 and SMRT promoted tamoxifen-induced cell cycle progression and proliferation [52]. In a breast cancer mouse model, reduced expression levels of NCoR1 correlate with the development of tamoxifen resistance [53]. In a cohort of ERα-positive unilateral invasive primary breast tumors from 99 postmenopausal patients who only received tamoxifen as adjuvant hormone therapy after primary surgery, low NCoR1 expression was associated with a significantly shorter relapse-free survival [54], substantiating a role of corepressor NCoR1 in tamoxifen resistance. In further support of this notion, based on ChIP analysis, over-expression of coactivator SRC-1 or corepressor SMRT is sufficient to alter the transcriptional action of tamoxifen on a number of target genes in breast and endometrial cancer cells [55].

Recent data reveal another mechanism of tamoxifen resistance caused by increased SRC-3 level. It is shown that the paired box 2 gene product (PAX2) is a tamoxifen-recruited transcriptional repressor of the ERBB2 gene. Increased SRC-3 can compete with PAX2 for ERRB2 gene binding and result in overexpression of ERRB2 and cause subsequent tamoxifen-resistant cell growth [56].

The importance of coregulators in determination of tamoxifen activity is also true for other SERMs. For instance, raloxifene promotes ERα interaction with NCoR1 both *in vivo* and *in vitro* [42]. In rat uterus, ormeloxifene antagonizes *in vivo* ERα activity by increasing the recruitment of NCoR1 corepressor and reducing the recruitment of SRC-1 coactivator [57]. In addition to SRCs, other coactivators can also play a role in SERM activity. For example, recruitment of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1β with ER LBD induced by tamoxifen contributes to the agonistic activity of tamoxifen in cultured cells [58].

6. SERMs regulate coregulator expression level and activities

Coregulators determine the SERM activity. On the other hand, SERMs can regulate the expression levels of coregulators, forming a feedback regulatory loop. For instance, in cultured cells, 4-hydroxytamoxifen and raloxifene increased protein stability and function of SRC-1 and SRC-3 coactivators in an ERα-dependent manner [59]. The increased coactivators subsequently enhance the transcriptional activity of other nuclear receptors, suggesting that these SERMS have broad biological actions in the cells [59]. In human patients, tamoxifen therapy leads to significantly increased expression of coactivators, particularly SRC-3, in both normal and malignant breast tissues [60]. In human skeletal muscle cells, tamoxifen and raloxifene increase SRC-1 mRNA levels while decreasing SMRT mRNA levels [61].

SERMs not only impact coactivator protein stability, but also can regulate the coactivator activity though posttranslational modifications such as phosphorylation. In response to anti-estrogen treatment, multiple growth signaling pathways are up-regulated (HER2, PI3K, PKC, etc). Activated kinases phosphorylate SRC-3,

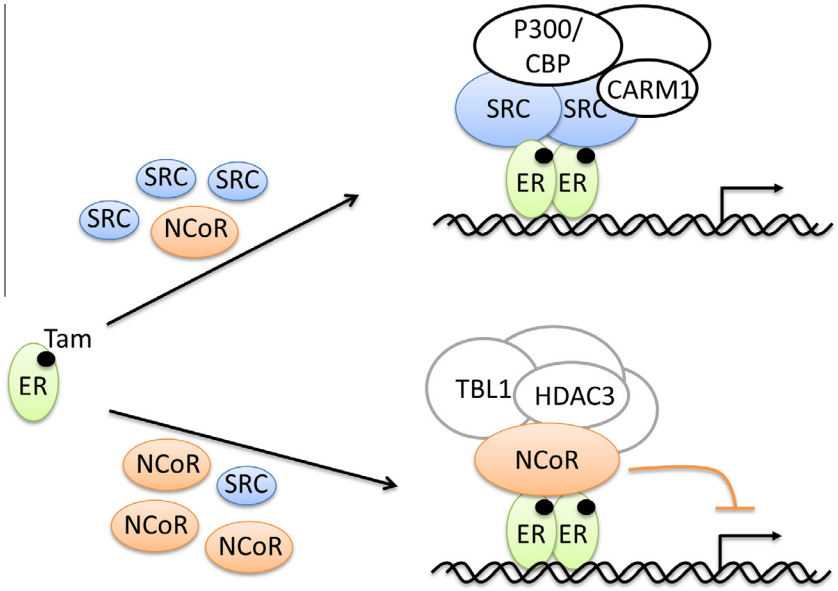

Fig. 2. The selective recruitment of coactivators and corepressors determines the SERM activity. Shown in the diagram is one representative mechanism in which the relative abundance of SRCs and NCoRs governs the recruitment. Other mechanisms could also influence recruitment, such as increased SRC recruitment by PKA-induced phosphorylation (See text for details).

enhance its coactivator activity, affect cell growth, and eventually contribute to resistance [62,63].

7. Summary

Since the cloning of the first nuclear receptor coactivator SRC-1 in 1995 [11], the last two decades have seen explosive growth of the coregulator field, with total numbers of about 450 coregulators characterized to date. Numerous studies have established that coregulators play pleiotropic roles in animal pathophysiology. In this review, we focused on the critical role of coregulators in SERM function since the literature reveals growing evidence to support that SERM activity is mainly determined by the selective recruitment of coactivators and corepressors to ER target gene in specific types of cells and tissues (Fig. 2). Better understanding of coactivator and corepressor functions should enhance future development of new generations of SERMs which have more beneficial and less harmful effects.

References

[1] Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–86.
[2] Della Torre S, Benedusi V, Fontana R, Maggi A. Energy metabolism and fertility – a balance preserved for female health. Nat Rev Endocrinol 2014;10:13–23.
[3] Maggi A, Ciana P, Belcredito S, Vegeto E. Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu Rev Physiol 2004;66:291–313.
[4] Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative group on hormonal factors in breast cancer. Lancet 1997;350:1047–59.
[5] Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 2003;362:419–27.
[6] Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
[7] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discovery 2003;2:205–13.
[8] Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995;270:1491–4.
[9] Rajbhandari P, Finn G, Solodin NM, Singarapu KK, Sahu SC, Markley JL, et al. Regulation of estrogen receptor alpha N-terminus conformation and function by peptidyl prolyl isomerase Pin1. Mol Cell Biol 2012;32:445–57.
[10] Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
[11] Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270:1354–7.
[12] Johnson AB, O'Malley BW. Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. Mol Cell Endocrinol 2012;348:430–9.
[13] Louie MC, Zou JX, Rabinovich A, Chen HW. ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. Mol Cell Biol 2004;24:5157–71.
[14] Al-azawi D, Ilroy MM, Kelly G, Redmond AM, Bane FT, Cocchiglia S, et al. Ets-2 and p160 proteins collaborate to regulate c-Myc in endocrine resistant breast cancer. Oncogene 2008;27:3021–31.
[15] Na SY, Lee SK, Han SJ, Choi HS, Im SY, Lee JW. Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated transactivations. J Biol Chem 1998;273:10831–4.
[16] Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 2009;9:615–30.
[17] Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997;387:733–6.
[18] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
[19] Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997;90:569–80.
[20] Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, et al. Regulation of transcription by a protein methyltransferase. Science 1999;284:2174–7.
[21] Voegel JJ, Heine MJ, Tini M, Vivat V, Chambon P, Gronemeyer H. The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. EMBO J 1998;17:507–19.
[22] Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, et al. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 1997;389:194–8.
[23] Zwart W, Theodorou V, Kok M, Canisius S, Linn S, Carroll JS. Oestrogen receptor-co-factor-chromatin specificity in the transcriptional regulation of breast cancer. EMBO J 2011;30:4764–76.
[24] Karmakar S, Foster EA, Smith CL. Unique roles of p160 coactivators for regulation of breast cancer cell proliferation and estrogen receptor-alpha transcriptional activity. Endocrinology 2009;150:1588–96.
[25] Malovannaya A, Lanz RB, Jung SY, Bulynko Y, Le NT, Chan DW, et al. Analysis of the human endogenous coregulator complexome. Cell 2011;145:787–99.
[26] Foulds CE, Feng Q, Ding C, Bailey S, Hunsaker TL, Malovannaya A, et al. Proteomic analysis of coregulators bound to ERalpha on DNA and nucleosomes reveals coregulator dynamics. Mol Cell 2013;51:185–99.
[27] Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 1998;279:1922–5.

[28] Mukherjee A, Soyal SM, Fernandez-Valdivia R, Gehin M, Chambon P, Demayo FJ, et al. Steroid receptor coactivator 2 is critical for progesterone-dependent uterine function and mammary morphogenesis in the mouse. Mol Cell Biol 2006;26:6571–83.
[29] Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci USA 2000;97:6379–84.
[30] Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.
[31] Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 2004;6:263–74.
[32] Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 1995;377:454–7.
[33] Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 1995;377:397–404.
[34] Hu X, Lazar MA. The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 1999;402:93–6.
[35] Perissi V, Staszewski LM, McInerney EM, Kurokawa R, Krones A, Rose DW, et al. Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev 1999;13:3198–208.
[36] Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, et al. Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev 1999;13:3209–16.
[37] Guenther MG, Lane WS, Fischle W, Verdin E, Lazar MA, Shiekhattar R. A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. Genes Dev 2000;14:1048–57.
[38] Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, et al. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 1997;89:373–80.
[39] Wen YD, Perissi V, Staszewski LM, Yang WM, Krones A, Glass CK, et al. The histone deacetylase-3 complex contains nuclear receptor corepressors. Proc Natl Acad Sci USA 2000;97:7202–7.
[40] Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 1997;11:657–66.
[41] Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB. The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol Endocrinol 1997;11:693–705.
[42] Webb P, Nguyen P, Kushner PJ. Differential SERM effects on corepressor binding dictate ERalpha activity in vivo. J Biol Chem 2003;278:6912–20.
[43] Yamamoto Y, Wada O, Suzawa M, Yogiashi Y, Yano T, Kato S, et al. The tamoxifen-responsive estrogen receptor alpha mutant D351Y shows reduced tamoxifen-dependent interaction with corepressor complexes. J Biol Chem 2001;276:42684–91.
[44] Jiang S, Meyer R, Kang K, Osborne CK, Wong J, Oesterreich S. Scaffold attachment factor SAFB1 suppresses estrogen receptor alpha-mediated transcription in part via interaction with nuclear receptor corepressor. Mol Endocrinol 2006;20:311–20.
[45] Nguyen TA, Hoivik D, Lee JE, Safe S. Interactions of nuclear receptor coactivator/corepressor proteins with the aryl hydrocarbon receptor complex. Arch Biochem Biophys 1999;367:250–7.
[46] Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002;295:2465–8.
[47] Scott DJ, Parkes AT, Ponchel F, Cummings M, Poola I, Speirs V. Changes in expression of steroid receptors, their downstream target genes and their associated co-regulators during the sequential acquisition of tamoxifen resistance in vitro. Int J Oncol 2007;31:557–65.
[48] Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003;95:353–61.
[49] Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance. increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.
[50] Su Q, Hu S, Gao H, Ma R, Yang Q, Pan Z, et al. Role of AIB1 for tamoxifen resistance in estrogen receptor-positive breast cancer cells. Oncology 2008;75:159–68.
[51] Zwart W, Griekspoor A, Berno V, Lakeman K, Jalink K, Mancini M, et al. PKA-induced resistance to tamoxifen is associated with an altered orientation of ERalpha towards co-activator SRC-1. EMBO J 2007;26:3534–44.
[52] Keeton EK, Brown M. Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT. Mol Endocrinol 2005;19:1543–54.
[53] Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, et al. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci USA 1998;95:2920–5.
[54] Girault I, Lerebours F, Amarir S, Tozlu S, Tubiana-Hulin M, Lidereau R, et al. Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clin Cancer Res 2003;9:1259–66.
[55] Romano A, Adriaens M, Kuenen S, Delvoux B, Dunselman G, Evelo C, et al. Identification of novel ER-alpha target genes in breast cancer cells: gene- and cell-selective co-regulator recruitment at target promoters determines the response to 17beta-estradiol and tamoxifen. Mol Cell Endocrinol 2010;314:90–100.
[56] Hurtado A, Holmes KA, Geistlinger TR, Hutcheson IR, Nicholson RI, Brown M, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. Nature 2008;456:663–6.
[57] Daverey A, Saxena R, Tewari S, Goel SK, Dwivedi A. Expression of estrogen receptor co-regulators SRC-1, RIP140 and NCoR and their interaction with estrogen receptor in rat uterus, under the influence of ormeloxifene. J Steroid Biochem Mol Biol 2009;116:93–101.
[58] Kressler D, Hock MB, Kralli A. Coactivators PGC-1beta and SRC-1 interact functionally to promote the agonist activity of the selective estrogen receptor modulator tamoxifen. J Biol Chem 2007;282:26897–907.
[59] Lonard DM, Tsai SY, O'Malley BW. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. Mol Cell Biol 2004;24:14–24.
[60] Haugan Moi LL, Hauglid Flageng M, Gandini S, Guerrieri-Gonzaga A, Bonanni B, Lazzeroni M, et al. Effect of low-dose tamoxifen on steroid receptor coactivator 3/amplified in breast cancer 1 in normal and malignant human breast tissue. Clin Cancer Res 2010;16:2176–2186.
[61] Dieli-Conwright CM, Spektor TM, Rice JC, Todd Schroeder E. Oestradiol and SERM treatments influence oestrogen receptor coregulator gene expression in human skeletal muscle cells. Acta Physiol (Oxf) 2009;197:187–196.
[62] Wu RC, Qin J, Yi P, Wong J, Tsai SY, Tsai MJ, et al. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic reponses to multiple cellular signaling pathways. Mol Cell 2004;15:937–49.
[63] Yi P, Wu RC, Sandquist J, Wong J, Tsai SY, Tsai MJ, et al. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). Mol Cell Biol 2005;25:9687–99.

Nuclear Receptor Coactivators: Master Regulators of Human Health and Disease

Subhamoy Dasgupta, David M. Lonard, and Bert W. O'Malley

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030; email: berto@bcm.edu

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Abstract

Transcriptional coregulators (coactivators and corepressors) have emerged as the principal modulators of the functions of nuclear receptors and other transcription factors. During the decade since the discovery of steroid receptor coactivator-1 (SRC-1), the first authentic coregulator, more than 400 coregulators have been identified and characterized, and deciphering their function has contributed significantly to our understanding of their role in human physiology. Deregulated expression of coregulators has been implicated in diverse disease states and related pathologies. The advancement of molecular technologies has enabled us to better characterize the molecular associations of the SRC family of coactivators with other protein complexes in the context of gene regulation. These continuing discoveries not only expand our knowledge of the roles of coactivators in various human diseases but allow us to discover novel coactivator-targeting strategies for therapeutic intervention in these diseases.

NR: nuclear receptor

SRC: steroid receptor coactivator

HAT: histone acetyltransferase

CARM: coactivator-associated arginine methyltransferase

INTRODUCTION

Transcriptional coregulators have emerged as the principal regulators of gene expression by directly interacting with and modulating the activity of essentially all nuclear receptors (NRs) and transcription factors. On the basis of their functional output, coregulators can be classified into two major classes: coactivators, which are associated with agonist-bound NRs to induce gene expression, and corepressors,which selectively repress gene expression through interaction with unliganded or antagonist-bound NRs (1, 2). Transcriptional induction is an ordered and dynamic process in which a NR first receives a signal from its cognate ligand and then translocates to specific DNA sequences in the promoter region of a target gene (3). This is followed by the recruitment of coactivator complexes, which then mediate enzymatic reactions, remodeling the chromatin and facilitating the association of the RNA polymerase II complex with the general transcriptional machinery at the target gene promoter. Because coregulators influence the transcriptional activity of numerous NRs and other types of transcription factors, they exert broad genome-wide effects on genetic networks and contribute significantly to a vast spectrum of physiological abnormalities and diseases (**Figure 1**) (4).

Since the discovery of steroid receptor coactivator-1 (SRC-1) a decade ago (5), more than 400 coregulators have been identified, and deciphering their function has significantly contributed to our understanding of coactivator biology. Gene-deletion studies in mice have demonstrated distinct roles for individual coregulators in growth and development and associated pathological states in genetic, reproductive, and metabolic disorders and cancer (**Figure 2**) (6). SRC family members of the p160 class of coactivators have been widely implicated in the regulation of steroid hormone action by mediating functions of a majority of the NRs and transcription factors, and their aberrant expression in different malignancies and genetic diseases has distinguished them as master regulators of human pathologies (7). Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) is another critical coregulator, which is involved in the regulation of metabolism and energy homeostasis. Expression levels of PGC-1α in various tissues have been associated with genetic predispositions to diseases with impaired mitochondrial function, including type 2 diabetes mellitus and obesity (8). Other coactivators, such as CREB-binding protein (CBP)-p300, interact with virtually all transcription factors and regulate gene expression by relaxing chromatin structure at the target gene promoter through their intrinsic histone acetyltransferase (HAT) activity. Mutations in the CBP gene are linked to Rubinstein-Taybi syndrome, in which a deficiency of CBP HAT activity results in defective development and long-term memory formation (9). In addition to chromatin remodeling, coactivator-associated arginine methyltransferases (CARM1/PRMT4) also stimulate gene transcription by activation of NRs and other transcription factors in combination with the SRC family of coactivators (10). Thus, advances in coactivator biology have significantly enhanced our basic understanding of the underlying molecular causes of various human diseases, and this understanding can now can be translated to the clinic through the discovery of novel coactivator-targeting therapeutics. This review outlines recent advances in coactivator biology and their implications in human health and disease, focusing primarily on SRC family members.

STRUCTURAL AND FUNCTIONAL DETERMINANTS OF SRC FAMILY MEMBERS

SRCs were the first of the gene families to be classified and characterized as coactivators for NRs. The SRC family consists of three members: SRC-1 (also known as NCOA1), SRC-2 (also known as NCOA2, GRIP1, and TIF2) and SRC-3 (also known as NCOA3, ACTR, AIB1, p/CIP, RAC3, and TRAM-1). The three members of the SRC family contain homologous domains and share sequence

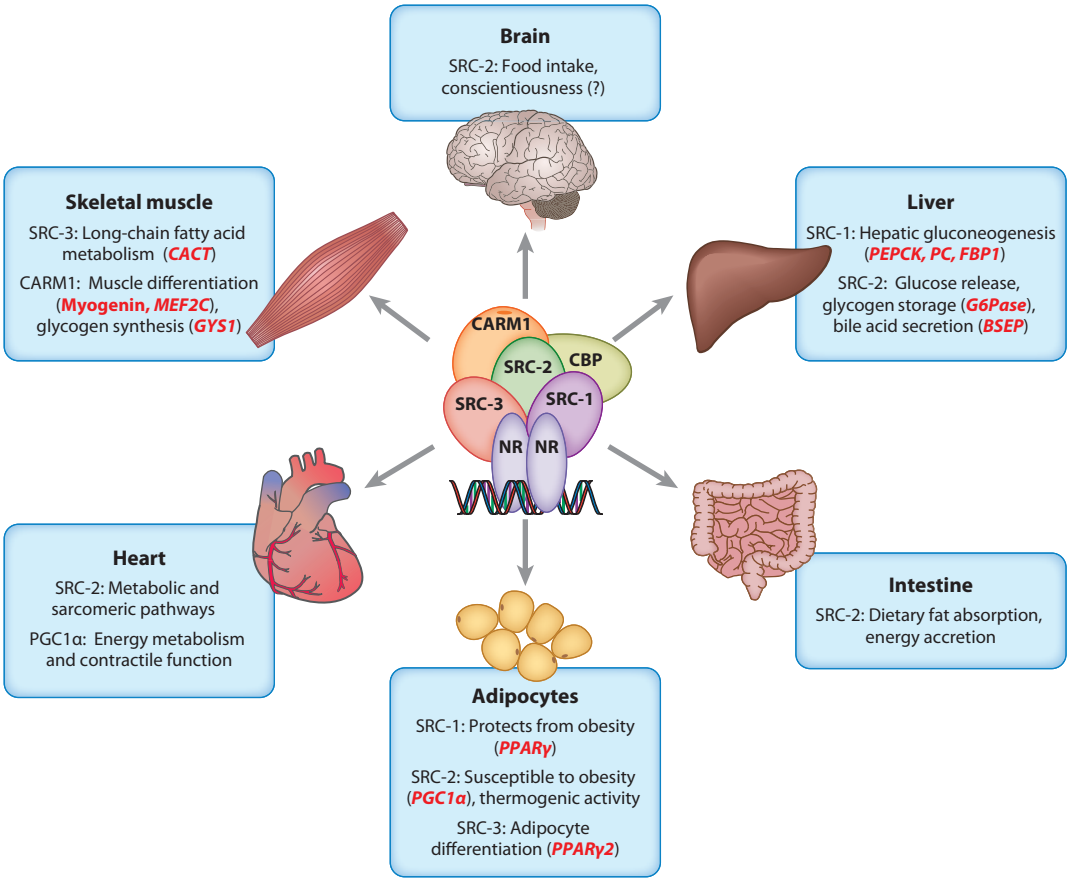

Figure 1

Nuclear receptor coactivators control the physiology of multiple organs that are critical for various metabolic disorders and some rare genetic diseases as well. Target genes transcriptionally regulated by the coactivators are indicated in red.

similarity—54% similarity between SRC-1 and -2, 50% between SRC-1 and -3, and 55% between SRC-2 and -3 (11, 12). Each member of the SRC family can enhance transcriptional activity of NRs by acting as a bridging molecule, assisting protein-protein interactions between NRs and multiple other coregulators and facilitating the assembly of the transcriptome complex at the target gene promoter. SRCs contain three distinct structural domains. These include the N-terminal basic helix-loop-helix-Per/ARNT/Sim (b-HLH-PAS); the central NR interacting domain, containing three LXXLL motifs (where X is any amino acid); and two C-terminal activation domains, AD1 and AD2. The N-terminal b-HLH-PAS domain contains the most conserved motif among SRC family members. It can interact with several transcription factors, such as STAT6, TEF4, and p53, and with additional coregulators, such as coiled-coil coactivator (CoCoA), flightless-I (Fli-I), and Pin1, to activate transcription (13). Additionally, the b-HLH-PAS domain possesses a bipartite nuclear localization signal (NLS), and a NLS-mutant coactivator fails to localize to the nucleus and is insensitive to proteasome-dependent turnover, an important means to limit its transcriptional activity (14). The central three LXXLL motifs that form the amphipathic alpha-helices are

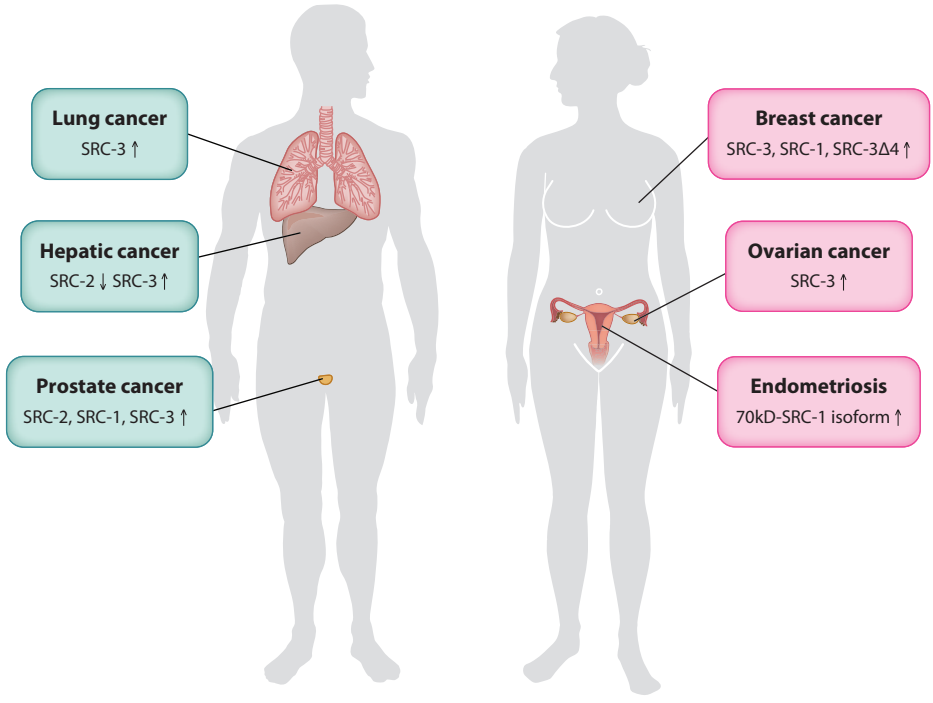

Figure 2

Steroid receptor coactivators (SRC family members) are abundantly expressed and amplified in various forms of cancer.

responsible for direct interaction and binding to the ligand-binding domain of NRs. The LXXLL motif of the coactivators fits into the hydrophobic cleft in the ligand-binding domain of the ligand-bound NR, and this association is facilitated by adjacent amino acid sequences. The C-terminal activation domains AD1 and AD2 are responsible for recruiting several additional cocoregulators for chromatin remodeling of the gene promoter to potentiate active transcription (15). SRCs recruit chromatin-modifying enzymes such as CBP and HAT by directly interacting with the AD1 domain, whereas AD2 binds to several important histone methyltransferases such as CARM1 and Protein arginine N-methyl transferase 1 (PRMT1). In addition to their functions as adaptor molecules for recruiting several chromatin-modifying enzymes to the DNA promoter, SRCs have weak intrinsic HAT activity at the C-terminal domain (16). These different structural features make SRCs critical and diverse platforms for the assembly and coordination of a variety of coregulators and transcription factors at the gene promoter and for the modulation of NR-dependent transcriptional activity.

POSTTRANSLATIONAL MODIFICATIONS

Although SRCs initially were identified as bona fide coactivators for NR-dependent transcription, they also interact with many different transcription factors and potentiate their transcriptional activity. These include nuclear factor–κB (NF-κB), E2F1, Smads, hypoxia inducible factor 1 (HIF1), p53, signal transducers and activators of transcription (STATs), the ETS (E-twenty-six) family, and HNF4 (hepatocyte nuclear factor-4) (15). Recent advances in proteomic applications identified several factors associated with an endogenous coactivator complexome that includes building blocks,

functional blocks, and regulatory subunits (17). Associations of these protein complexes are highly ordered processes that are regulated by SRCs in response to a variety of upstream extracellular stimuli to orchestrate selective upregulation of target genes. These precise genetic regulations by SRCs in response to signaling inputs are controlled by enzymes that impose posttranslational modification (PTM) codes on the coactivators. Different combinatorial PTM codes on SRCs alter their structural conformation and affinity toward specific transcription-complex proteins, promoting upstream-signal-specific genetic changes to execute physiological programs. The diverse sets of enzymes that interact with and modify SRCs include kinases, phosphatases, small ubiquitin-related modifier (SUMO) ligases, ubiquitin ligases, HATs, and histone methyltransferases (18). Several extracellular stimuli such as steroid hormones, growth factors such as epidermal growth factor (EGF) and heregulin, and cytokines transduce downstream signaling events, generating phosphorylation codes on SRCs. Particularly, mitogen-activated protein kinase (MAPK) phosphorylates SRC-1 at Thr^{1179} and Ser^{1185} and SRC-2 at Ser^{736}, increasing coactivator affinity toward the androgen receptor (AR), estrogen receptor–alpha (ERα), and progesterone receptor (PR). Phospho-coded SRCs also have higher binding affinity for p300 and CBP, thereby enhancing their recruitment to the chromatin and activating NR-dependent transcription in a ligand-independent manner. Protein kinase A (PKA) has been documented to phosphorylate SRC-2, increasing its turnover and nuclear translocation, followed by rapid degradation by the ubiquitin-proteasome pathway (19).

Recent studies from our laboratory identified SRC-2 as a direct substrate of AMP-activated protein kinase (AMPK), and AMPKα2-subunit-dependent phosphorylation of SRC-2 enhances the activity of transcription factor farnesoid X receptor (FXR) on target gene promoters (20). Among the SRC family members, SRC-3 is the most attractive substrate for multiple kinases, and the functional importance of these modifications in transcriptome assembly and coactivator biology has been extensively studied. Work from our laboratory and others has identified several sites (Thr^{24}, Ser^{505}, Ser^{543}, Ser^{601}, Ser^{857}, Ser^{860}, and Ser^{867}) in the Ser-Thr-rich region of SRC-3 that are frequently phosphorylated by multiple kinases such as MAPK, inhibitor of kappa B kinase (IKK), protein kinase C ζ (PKCζ), Akt, focal adhesion kinase (FAK), casein kinase 1 isoforma delta, and extracellular-signal regulated kinase 3 (ERK3) (21–28). Phosphorylation of SRC-3 selectively increases its affinity for certain transcription factors and NRs, thereby potentiating robust transcriptional activity on target gene promoters. In addition to Ser-Thr kinases, the tyrosine kinase ABL phosphorylates SRC-3 on Tyr^{1357}, and this modification promotes rapid recruitment of p300 and CBP along with strong coactivation of NRs and NFκB (29). These findings illustrate how PTM codes on SRC-3 allow it to accept signals from multiple pathways and to delicately coordinate biological functions by modulating specific genetic changes.

Combinations of PTM codes on SRC-3 further alter the nature and stability of the coactivator and supplement additional parameters that regulate its activity. Phosphorylation-dependent ubiquitination and regulation of SRC-3 protein turnover have identified intricately regulated combinations of PTM codes that modulate coactivator function. On one hand, phosphorylation of SRC-3 by glycogen synthase kinase 3 (GSK3β) increases transcriptional activity by promoting subsequent coactivator degradation by ubiquitination, while on the other hand PKCζ-mediated SRC-3 phosphorylation blocks its ubiquitination and increases its levels by hindering its proteasome-mediated degradation (30). In addition to ubiquitination, certain SRC family members are direct targets of SUMO ligases, and this modification sometimes competes with ubiquitinylation for the same lysine residue, which can alter the cellular fate of SRCs by blocking protein degradation while diminishing its transcriptional

PTM: posttranslational modification

SUMO ligase: small ubiquitin-related modifier ligase

AR: androgen receptor

TNFα: tumor necrosis factor–alpha

MMP-9: matrix metalloproteinase–9

EMT: epithelial-mesenchymal transition

activity (15). Thus, combinatorial PTM codes on SRC-3 determine the coactivator concentration and the gene-selective potency that allows it to control signal-specific gene expression.

The activities of several coactivators other than SRC family members are regulated by PTMs. For instance, phosphorylation of a conserved Ser^{217} residue in CARM1 functions as a molecular switch leading to CARM1 activation (16). Additionally, an automethylation domain has been identified in CARM1, and this self-regulatory mark modulates CARM1-activated transcription and pre-mRNA splicing without affecting its enzymatic activity (31).

THE ROLE OF NUCLEAR RECEPTOR COACTIVATORS IN HUMAN DISEASE AND PATHOLOGY

Many of the functions of coactivators, particularly of the SRC family members, that we understand today were discovered in classical mouse genetic studies that demonstrate exclusive roles for each SRC in reproduction, cancer, and metabolic homeostasis.

Reproductive Functions

SRCs are dominant coactivators involved in reproductive physiology regulating uterine growth, embryo implantation, and fertility. Disruption of the SRC-1 gene in mice resulted in decreased growth and development of reproductive organs without eliminating viability and fertility. Loss of the SRC-1 gene significantly reduced uterine, prostatic, testicular, and mammary gland growth in response to steroid hormone stimulation. SRC-1 ablation decreased uterine growth by 60% and is recognized as the most potent coactivator for ER- and PR-dependent functions in the uterus (32). In contrast, SRC-3 is the primary coactivator for ER and PR in mammary luminal epithelial cells (33, 34). Additionally, SRC-3 knockout mice exhibit pleiotropic processes including postnatal growth retardation, delayed puberty, abnormal reproductive physiology, and reduced mammary-ductal branching and alveologenesis (34). SRC-2 gene global knockouts show striking abnormalities in reproductive functions of both sexes. Male SRC-2 knockouts are hypofertile owing to defects in spermatogenesis and testicular degeneration, whereas female hypofertility is attributed to placental hypoplasia, a block in embryo implantation, and a defective decidual response (35). In addition, loss of the SRC-2 gene in $PR^{Cre/+}$ bigenic mice resulted in reduced mammary-ductal side branching and alveologenesis, suggesting an essential role for SRC-2 in progesterone-induced mammary morphogenesis (36). Although single-knockout mice for each SRC family member are viable, the phenotypes observed in double-knockout mice are lethal. This indicates a degree of physiological cooperation among SRC family members compensating in part for defects in the functional output.

In humans, SRCs are altered in endometriosis, a disease in which cells from the lining of the uterus during retrograde menses survive and grow outside the abdominal cavity. Although immunohistochemical analysis demonstrated endometriotic tissue–specific expression of all SRCs, full-length SRC-1 is expressed at lower levels than in normal endometrium (37). Recent studies from our laboratory uncovered a novel signaling axis involving SRC-1 in an endometriosis mouse model and biopsied stromal cells from human patients. We identified reduced expression of full-length SRC-1 in eutopic and ectopic endometrium of mice with a concomitant increase in a 70-kD SRC-1 proteolytic isoform compared to normal endometrium. Mechanistic studies revealed that tumor necrosis factor α (TNFα)-mediated matrix metalloproteinase-9 (MMP-9) activation cleaves full-length SRC-1 to generate the 70-kD isoform, which in turn blocks TNFα-induced apoptosis and promotes cellular invasion and epithelial-mesenchymal transition (EMT) (38). This newly discovered SRC-1 isoform has strong clinical implications for endometriosis patients and potentially can be used for diagnostic and therapeutic purposes (39). Other ER coactivators have been implicated as a risk factor for endometrial

cancer. Studies have identified cumulative genetic variations of the SRC-2 cofactor complexosome as a whole, including SRC-2, CARM1, p300, CBP, and PRMT1, as a significant risk associated with endometrial cancer (40).

Energy Metabolism

NR coactivators also have emerged as primary regulators of metabolic homeostasis in different tissues. Global-knockout mouse models deleting each SRC family member, as well as tissue-specific deletion of SRCs, have identified important metabolic pathways that they regulate. Understanding these pathways has broadened our knowledge of the molecular mechanisms of metabolic disorders such as diabetes, obesity, and certain genetic disorders. For instance, whereas SRC-1$^{-/-}$ mice are prone to obesity owing to decreased energy expenditure, SRC-2$^{-/-}$ mice are protected from high-fat-induced obesity, suggesting a dynamic interplay of the coactivators regulating normal metabolic physiology (41). SRC-2 knockout mice display increased insulin sensitivity, higher lipolysis, and decreased fat uptake in conjunction with enhanced adaptive thermogenesis by modulating brown adipose tissue (BAT) activity. Molecularly, loss of SRC-2 decreases peroxisome proliferator-activated receptor (PPAR) activity in white adipose tissue (WAT), whereas it induces interaction of SRC-1 with PGC1α in BAT, thereby promoting thermogenic activity. In part, these mechanisms address the obesity-resistant phenotype observed in SRC-2$^{-/-}$ mice (41). In addition to the roles of SRC-1 and SRC-2 in energy metabolism, SRC-3 is an important coordinator of white adipocyte development, and SRC-3$^{-/-}$ mice show impaired adipocyte formation (42, 43). Mechanistically, SRC-3 coactivates transcription factor CAAT/enhancer-binding protein to induce expression of PPARγ2, promoting adipogenesis. Loss of SRC-3 thus impairs the white adipogenic program (44). Similarly, SRC-1/SRC-3 double-knockout animals exhibit defective adaptive thermogenesis and low BAT activity due to downregulation of selective PPARγ target genes. These animals are lean compared to their wildtype littermates and are resistant to diet-induced obesity (45). Collectively, SRC family members have emerged as gatekeepers of energy homeostasis, maintaining the balance between BAT and WAT.

In addition to their distinct roles in adipogenesis, SRC family members display important phenotypic effects in muscle and liver energy metabolism. Global metabolomic profiling of SRC knockout animals identified common metabolites that are statistically altered in different SRC family members (46). Interestingly, SRC-3 knockout mice display an increase in long-chain acyl carnitine in skeletal muscle due to reduced expression of the long-chain fatty acid transporter carnitine/acyl-carnitine translocase (CACT). As a result of this genetic discrepancy, SRC-3$^{-/-}$ mice resemble the CACT-deficiency phenotype observed in humans, characterized by a syndrome of hypoketonemia, hypoglycemia, hyperammonemia, and impaired neurologic, cardiac, and skeletal muscle performance (46). Targeted deletion of SRC-2 in skeletal muscle increases mitochondrial uncoupling in muscle myocytes by modulating the expression of the UCP3 gene with a simultaneous increase in energy expenditure, thus delaying the development of type 2 diabetes (47). CARM1 plays a pivotal role in myogenesis and muscle differentiation in conjunction with SRC-2. CARM1 physically interacts with SRC-2 and is responsible for the recruitment and activation of the MEF2 transcription factor on the creatine kinase promoter. Inhibition of CARM1 significantly abrogates muscle differentiation and decreases expression of the important muscle transcription factors myogenin and MEF2 (48). Metabolically, CARM1 is associated with muscle glycogen synthesis by directly regulating transcription of *Gys1* (glycogen synthase 1), *Pgam2* (muscle phosphoglycerate mutase 2), and *Pygm* (muscle glycogen phosphorylase) genes. Attenuation of CARM1 significantly reduces muscle mass, suggesting it plays a role in human glycogen-storage diseases (49).

Loss of SRC-2 also affects hepatic glucose release owing to reduced expression of glucose-6-phosphatase (G6Pase). As a result of this genetic defect, SRC-2$^{-/-}$ mice show fasting hypoglycemia and resemble the phenotype observed in human glycogen-storage disorder-1a (Von Gierke's disease). Molecularly, SRC-2 strongly coactivates the orphan NR RORα on the G6Pase promoter to regulate G6Pase transcription and hepatic glucose release (50). SRC-1$^{-/-}$ mice exhibit a hypoglycemic condition in both fasting and fed states, and this is partly due to impairment of the gluconeogenic program. SRC-1 directly coordinates rate-limiting enzymes of the gluconeogenic pathway such as pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), and fructose-1, 6-bisphosphatase (FBP1), thus restoring normal glucose levels in fasting conditions (51).

Recent findings from our laboratory also identified SRC-2 as an essential coordinator of whole-body energy homeostasis. It acts by regulating the absorption of dietary fat and energy utilization. Reduced energy status activates the fuel-sensor kinase AMPK, which then phosphorylates and activates SRC-2. Activated SRC-2 stimulates the action of the FXR transcription factor on the bile salt export pump (BSEP) promoter, thereby increasing its gene transcription. BSEP causes secretion of bile acid, allowing absorption of dietary fats and triglycerides, which are then circulated to various organs for restoration of energy. As expected, both global SRC-2 knockout mice and liver-specific SRC-2 deletion phenocopies increased fecal triglycerides and decreased plasma triglycerides, suggesting that loss of SRC-2 attenuates absorption of dietary fats (20). Based on these observations, it is reasonable to speculate that the AMPK-SRC-2 axis serves as a potential therapeutic target to modulate whole-body energy homeostasis (52). Ablation of SRC-2 also affects the metabolic program in the adult heart with impaired sarcomeric gene expression, mimicking a stressed heart phenotype. These studies conclude that SRC-2 acts as a pivotal transcriptional regulator in the adult heart and that it is required for normal functioning during pressure overload (53). Recent studies from SRC-1 and SRC-3 double-knockout mice found that the dual loss of these coactivators enables resistance to age-related obesity and glucose intolerance by the upregulation of insulin receptor substrate 1 (IRS1) (54). Taken together, these findings emphasize the importance of SRCs as essential coordinators of energy metabolism and highlight their clinical implications in various metabolic and genetic disorders.

Cancer Progression and Metastasis

Each member of the SRC family is overexpressed and/or amplified in different types of cancers (reviewed in Reference 15). In particular, SRCs have been widely implicated in endocrine-related cancers such as breast, prostate, endometrial, and ovarian cancers. In breast cancer, SRC-3 and SRC-1 genes are frequently amplified and/or overexpressed, and their expression positively correlates with poor prognosis and more advanced disease stage. Molecularly, SRC-1 promotes breast-to-lung metastasis by upregulating Twist expression and coactivating the polyoma enhancer activator 3 (PEA3) transcription factor, thereby promoting migration, invasion and EMT (55). In addition to its established role in tamoxifen-resistant breast cancer, SRC-1 has been implicated in aromatase inhibitor (AI)-resistant recurrent breast cancer (56). Resistant patients exhibit increased expression of SRC-1, which correlates with poor disease-free survival and distant organ metastasis. This is achieved partly by direct interactions with the ETS2 transcription factor and induction of the downstream target genes *MYC* and *MMP9*, which are responsible for the invasive and migratory phenotypes seen in AI-resistant breast cancer cells (56). Thus, SRC-1 has been proposed to be a dynamic coactivator that can switch breast tumor progression from a steroid-responsive to a steroid-resistant state. SRC-1 transcriptionally enhances a critical protease, ADAM22, responsible for aggressive phenotypes in breast cancer cells independent of ER. Also, integrin-α(5)

(ITGA5) is a direct target of SRC-1 in ER-negative breast tumors; it controls a cascade of downstream signals regulating cell adhesion (57). These findings vividly demonstrate that SRC-1 can promote aggressive breast tumor phenotype and suggest that therapeutic intervention targeting SRC-1 should be advantageous to breast cancer patients.

SRC-3 has been extensively studied in various breast cancer mouse models because of its crucial role in tumor growth, cellular proliferation, EMT, and invasiveness (15). Importantly, SRC-3 serves as a downstream master effector molecule of the Her-2 signaling axis, and loss of SRC-3 significantly attenuates Her-2-dependent breast tumor progression (58). Multiple kinases tend to modulate SRC-3 activity through various PTM codes, demonstrating the global role of SRC-3 in integrating upstream signaling events to promote aggressive progression in breast cancer. In addition to ER and PR, SRC-3 functionally activates other breast oncogenic transcription factors such as NFκB, PEA3, and IKK, and facilitates transcriptional upregulation of cancer-specific genes (21, 22, 27). Common downstream targets affected by SRC-3 activation include the insulin-like growth factor-1 (IGF1) pathway, cyclin D1, and MMPs (15). Recent studies have identified a novel cofactor, PELP1, which plays an important role in ERα signaling by directly interacting with CARM1. PELP1 is a proto-oncogene aberrantly expressed in breast cancer, and its interaction with CARM1 synergistically enhances ERα transactivation. Interestingly, PELP1 and CARM1 were found to be co-overexpressed in a subset of ER-positive breast cancers and were suggested as attractive therapeutic targets (59). CARM1 has also been identified to be a dominant coactivator in a variety of cancers, including colon cancer, where it was found to coactivate β-catenin downstream of Wnt signaling. Depletion of CARM1 significantly reduced Wnt-induced target gene expression, thereby suppressing tumor cell growth and survival (60).

SRC-3 has been implicated in other cancers as well. Recent evidence suggests increased copy-number alteration of SRC-3 in a subset of lung cancers (61). Studies from our laboratory identified SRC-3 to be a substrate of an atypical MAPK named ERK3, which signals SRC-3 to promote invasion-metastasis by enhancing expression of MMP genes in lung cancer cells (27). In prostate cancer, SRC-2 has been identified as a dominant oncogene amplified in ~8% of primary tumors and ~37% of metastatic patients (62). SRC-2 acts as a strong coactivator of AR, and promotes prostate cancer cell growth by activating cyclin D1 gene expression (63, 64). In addition, SRC-2 has AR-independent functions and can promote prostate cancer cell growth in the absence of AR. SRC-2 expression positively correlates with aggressive prostate cancer in patients, and its expression is a valuable indicator of time to disease recurrence (64). Several SRC-2 mutations have been identified in lung cancer, prostate cancer, and melanoma. The majority of these mutations (G435S, G439D, and P470S) have been identified in the Ser/Thr-rich domain, which is a direct target of several kinases (62). One of these mutations lies in the AD1 region responsible for recruitment of p300 and CBP; however, the functional significance of these mutations with respect to disease progression remains unknown. In prostate cancer, the cofactor PGC-1α also has been reported to be a strong coactivator of AR by directly interacting with the transactivation domain of AR. Depletion of PGC-1α significantly decreased AR-dependent prostate cancer cell growth by inducing a cell cycle G1 arrest, suggesting that PGC-1α could be a potential target for prostate cancer therapy (65). In a subset of acute myeloid leukemia (AML) patients, SRC-2 gene fusions with monocytic leukemia zinc finger protein (MOZ) have been detected (66). A gene rearrangement occurs between the 5′ of the MOZ gene and the 3′ of the SRC-2 gene, encoding the transcriptional activation domain of MOZ and the coactivator AD1 domain. This rearrangement enables the expressed fusion protein to recruit CBP, which drives AML (67). Recent findings also identified *SRC-1* as a potential driver gene in chromosome 2p that is frequently found to be

amplified in chronic lymphocytic leukemia (CLL). Gain of 2p chromosomal region function in CLL patients is detected at an early stage of the disease and correlates with poor prognosis, indicating SRC-1 may contribute to the pathological state of CLL (68). A recent study identified enhanced expression of the PGC-1α coactivator in a subset of human melanoma tumors. PGC-1α-positive melanoma cells exhibited increased mitochondrial activity and were resistant to reactive oxygen species stress (69).

In addition to the involvement of SRCs in cancer (70), recent evidence points to a requirement for coactivators in chemoresistance (71) as well as in stem cell self-renewal and pluripotency maintenance (72, 73). Under cytotoxic stress, SRC-3 deregulates p53 induction by upregulating TNF receptor–associated factor 4 (TRAF4), which competes with p53 for binding to the deubiquitinase HAUSP (herpesvirus-associated ubiquitin-specific protease). This ability of SRC-3 along with TRAF4 to destabilize p53 may be an important mechanism for chemoresistance to current breast cancer therapies. SRC-3 was also found to directly bind to the estrogen-related receptor β (Esrrb) and promote Esrrb transcriptional activity by recruiting CBP and CARM1 to regulate *Oct4*, *Sox2*, and the *Nanog* gene network (73). ChIPseq studies identified an overlapping regulatory network of SRC-3 with Esrbb supporting the Oct4-Sox2-Nanog transcriptional program to maintain embryonic stem cell (ESC) pluripotency and self-renewal (72). These studies propose a new concept in stem cell biology and suggest a dynamic interplay involving the SRC coactivators and the transcription factors in ESC maintenance. It will be interesting to decode other possible coactivator functions in cancer stem cell biology.

TARGETING STRATEGIES

Because the coactivators can integrate numerous upstream signaling events into functional outputs, they may be an attractive therapeutic target for the treatment of cancer. As opposed to NRs, which are relatively easy to target thanks to their affinity for ligands, coactivators are more difficult to target with small molecules (6). Approaches to block coactivator molecules include targeting the enzymes that posttranslationally modify the coactivators, thereby altering the stability of the proteins (74). However, recent high-throughput screening has identified small-molecule inhibitors that can target SRC-1 and SRC-3 by direct binding to these coactivators (75). Binding of small-molecule inhibitors to the oncogenic SRC-3 coactivator promotes its degradation and attenuates cancer cell growth. This proof-of-principle study laid the foundation for an ongoing screening effort.

CONCLUSION

Transcriptional coactivators have emerged as "master regulators" of human physiology, and their dysregulation has been implicated in numerous diseases (76). During evolution SRCs have emerged from strong selection pressures and are thus regarded as key factors for human adaptation (7). The involvement of SRC family members in reproduction, energy metabolism, and growth outlines the importance of this class of molecules in human physiology and disease. As metabolism and growth are essential for survival and multiplication, cancer cells often hijack and co-opt these multifaceted "master" molecules to enhance their proliferative behavior. Thus, understanding the role of SRCs in energy homeostasis of cancer cells should shed light on additional roles for coactivators in cancer biology. Environmental stresses such as fluctuations in nutrient availability and dietary composition lead to various phenotypes in mouse models of coactivator gene disruption, indicating that SRCs can orchestrate broad transcriptional reprograms to utilize available nutrients for the maintenance of energy homeostasis. Ongoing and future studies will expand our understanding of NR coactivators in various human pathologies and should identify additional targeting strategies for therapeutic interventions in multiple diseases.

SUMMARY POINTS

1. Transcriptional coactivators have emerged as the principal regulators of gene expression by directly interacting with and modulating the activity of essentially all nuclear receptors (NRs) and transcription factors.
2. SRCs were the first gene family to be classified and characterized as coactivators for NRs. Each member of the SRC family can enhance transcriptional activity of NRs by acting as a bridging molecule, assisting protein-protein interactions between NRs and multiple other coregulators, and facilitating the assembly of the transcriptome complex at target gene promoters.
3. Different combinatorial PTM codes on SRCs alter their conformation and affinity toward specific transcription-complex proteins to promote upstream-signal-specific genetic changes to execute physiological programs. Diverse sets of enzymes that interact with and modify SRCs include kinases, phosphatases, small ubiquitin-related modifier (SUMO) ligases, ubiquitin ligases, histone acetyltransferases, and histone methyltransferases.
4. NR coactivators have emerged as primary regulators of metabolic homeostasis, modulating energy balance in different tissues.
5. SRCs are often highly amplified and overexpressed in different types of malignancy, driving primary tumor growth, invasion-metastasis, and drug resistance.
6. Because they can integrate numerous upstream signaling events into functional outputs, coactivators are regarded as attractive therapeutic targets for the treatment of cancer.

DISCLOSURE STATEMENT

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LITERATURE CITED

1. Lonard DM, Kumar R, O'Malley BW. 2010. Minireview: the SRC family of coactivators: an entree to understanding a subset of polygenic diseases? *Mol. Endocrinol.* 24:279–85
2. O'Malley BW, Malovannaya A, Qin J. 2012. Minireview: nuclear receptor and coregulator proteomics—2012 and beyond. *Mol. Endocrinol.* 26:1646–50
3. Tsai MJ, O'Malley BW. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 63:451–86
4. Lonard DM, O'Malley BW. 2006. The expanding cosmos of nuclear receptor coactivators. *Cell* 125:411–14
5. Onate SA, Tsai SY, Tsai MJ, et al. 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270:1354–57
6. Lonard DM, O'Malley BW. 2012. Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nat. Rev. Endocrinol.* 8:598–604
7. York B, O'Malley BW. 2010. Steroid receptor coactivator (SRC) family: masters of systems biology. *J. Biol. Chem.* 285:38743–50
8. Puigserver P, Adelmant G, Wu Z, et al. 1999. Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* 286:1368–71

9. Petrij F, Giles RH, Dauwerse HG, et al. 1995. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature* 376:348–51
10. Chen D, Ma H, Hong H, et al. 1999. Regulation of transcription by a protein methyltransferase. *Science* 284:2174–77
11. Mukherjee A, Amato P, Allred DC, et al. 2007. Steroid receptor coactivator 2 is required for female fertility and mammary morphogenesis: insights from the mouse, relevance to the human. *Nucl. Recept. Signal* 5:e011
12. Johnson AB, O'Malley BW. 2012. Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. *Mol. Cell. Endocrinol.* 348:430–39
13. Hsia EY, Goodson ML, Zou JX, et al. 2010. Nuclear receptor coregulators as a new paradigm for therapeutic targeting. *Adv. Drug Deliv. Rev.* 62:1227–37
14. Li C, Wu RC, Amazit L, et al. 2007. Specific amino acid residues in the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-dependent turnover. *Mol. Cell. Biol.* 27:1296–308
15. Xu J, Wu RC, O'Malley BW. 2009. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. *Nat. Rev. Cancer* 9:615–30
16. Feng Q, He B, Jung SY, et al. 2009. Biochemical control of CARM1 enzymatic activity by phosphorylation. *J. Biol. Chem.* 284:36167–74
17. Malovannaya A, Lanz RB, Jung SY, et al. 2011. Analysis of the human endogenous coregulator complexome. *Cell* 145:787–99
18. Han SJ, Lonard DM, O'Malley BW. 2009. Multi-modulation of nuclear receptor coactivators through posttranslational modifications. *Trends Endocrinol. Metab.* 20:8–15
19. Borud B, Hoang T, Bakke M, et al. 2002. The nuclear receptor coactivators p300/CBP/cointegrator-associated protein (p/CIP) and transcription intermediary factor 2 (TIF2) differentially regulate PKA-stimulated transcriptional activity of steroidogenic factor 1. *Mol. Endocrinol.* 16:757–73
20. Chopra AR, Kommagani R, Saha P, et al. 2011. Cellular energy depletion resets whole-body energy by promoting coactivator-mediated dietary fuel absorption. *Cell Metab.* 13:35–43
21. Wu RC, Qin J, Hashimoto Y, et al. 2002. Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) coactivator activity by I kappa B kinase. *Mol. Cell. Biol.* 22:3549–61
22. Wu RC, Qin J, Yi P, et al. 2004. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic responses to multiple cellular signaling pathways. *Mol. Cell* 15:937–49
23. Yi P, Wu RC, Sandquist J, et al. 2005. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). *Mol. Cell. Biol.* 25:9687–99
24. Yi P, Feng Q, Amazit L, et al. 2008. Atypical protein kinase C regulates dual pathways for degradation of the oncogenic coactivator SRC-3/AIB1. *Mol. Cell* 29:465–76
25. York B, Yu C, Sagen JV, et al. 2010. Reprogramming the posttranslational code of SRC-3 confers a switch in mammalian systems biology. *Proc. Natl. Acad. Sci. USA* 107:11122–27
26. Long W, Yi P, Amazit L, et al. 2010. SRC-3Delta4 mediates the interaction of EGFR with FAK to promote cell migration. *Mol. Cell* 37:321–32
27. Long W, Foulds CE, Qin J, et al. 2012. ERK3 signals through SRC-3 coactivator to promote human lung cancer cell invasion. *J. Clin. Invest.* 122:1869–80
28. Li C, Liang YY, Feng XH, et al. 2008. Essential phosphatases and a phospho-degron are critical for regulation of SRC-3/AIB1 coactivator function and turnover. *Mol. Cell* 31:835–49
29. Oh AS, Lahusen JT, Chien CD, et al. 2008. Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by ABL kinase and is required for its activity in cancer cells. *Mol. Cell. Biol.* 28:6580–93
30. Wu RC, Feng Q, Lonard DM, et al. 2007. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* 129:1125–40
31. Kuhn P, Chumanov R, Wang Y, et al. 2011. Automethylation of CARM1 allows coupling of transcription and mRNA splicing. *Nucleic Acids Res.* 39:2717–26

32. Xu J, Qiu Y, DeMayo FJ, et al. 1998. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* 279:1922–25
33. Han SJ, DeMayo FJ, Xu J, et al. 2006. Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. *Mol. Endocrinol.* 20:45–55
34. Xu J, Liao L, Ning G, et al. 2000. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc. Natl. Acad. Sci. USA* 97:6379–84
35. Gehin M, Mark M, Dennefeld C, et al. 2002. The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. *Mol. Cell. Biol.* 22:5923–37
36. Mukherjee A, Soyal SM, Fernandez-Valdivia R, et al. 2006. Steroid receptor coactivator 2 is critical for progesterone-dependent uterine function and mammary morphogenesis in the mouse. *Mol. Cell. Biol.* 26:6571–83
37. Suzuki A, Horiuchi A, Oka K, et al. 2010. Immunohistochemical detection of steroid receptor cofactors in ovarian endometriosis: involvement of down-regulated SRC-1 expression in the limited growth activity of the endometriotic epithelium. *Virchows Arch.* 456:433–41
38. Han SJ, Hawkins SM, Begum K, et al. 2012. A new isoform of steroid receptor coactivator-1 is crucial for pathogenic progression of endometriosis. *Nat. Med.* 18:1102–11
39. Dyson MT, Bulun SE. 2012. Cutting SRC-1 down to size in endometriosis. *Nat. Med.* 18:1016–18
40. Li Y, Low HQ, Foo JN, et al. 2012. Genetic variants in ER cofactor genes and endometrial cancer risk. *PLoS One* 7:e42445
41. Picard F, Gehin M, Annicotte J, et al. 2002. SRC-1 and TIF2 control energy balance between white and brown adipose tissues. *Cell* 111:931–41
42. Louet JF, Coste A, Amazit L, et al. 2006. Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. *Proc. Natl. Acad. Sci. USA* 103:17868–73
43. Louet JF, O'Malley BW. 2007. Coregulators in adipogenesis: What could we learn from the SRC (p160) coactivator family? *Cell Cycle* 6:2448–52
44. Coste A, Louet JF, Lagouge M, et al. 2008. The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1α. *Proc. Natl. Acad. Sci. USA* 105:17187–92
45. Wang Z, Qi C, Krones A, et al. 2006. Critical roles of the p160 transcriptional coactivators p/CIP and SRC-1 in energy balance. *Cell Metab.* 3:111–22
46. York B, Reineke EL, Sagen JV, et al. 2012. Ablation of steroid receptor coactivator-3 resembles the human CACT metabolic myopathy. *Cell Metab.* 15:752–63
47. Duteil D, Chambon C, Ali F, et al. 2010. The transcriptional coregulators TIF2 and SRC-1 regulate energy homeostasis by modulating mitochondrial respiration in skeletal muscles. *Cell Metab.* 12:496–508
48. Chen SL, Loffler KA, Chen D, et al. 2002. The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. *J. Biol. Chem.* 277:4324–33
49. Wang SC, Dowhan DH, Eriksson NA, et al. 2012. CARM1/PRMT4 is necessary for the glycogen gene expression programme in skeletal muscle cells. *Biochem. J.* 444:323–31
50. Chopra AR, Louet JF, Saha P, et al. 2008. Absence of the SRC-2 coactivator results in a glycogenopathy resembling Von Gierke's disease. *Science* 322:1395–99
51. Louet JF, Chopra AR, Sagen JV, et al. 2010. The coactivator SRC-1 is an essential coordinator of hepatic glucose production. *Cell Metab.* 12:606–18
52. Ferre P, Foufelle F. 2011. A new role for a metabolic star: AMP-activated protein kinase stimulates fat absorption. *Cell Metab.* 13:1–2
53. Reineke EL, York B, Stashi E, et al. 2012. SRC-2 coactivator deficiency decreases functional reserve in response to pressure overload of mouse heart. *PLoS One* 7:e53395
54. Wang Z, Shah OJ, Hunter T. 2012. The transcriptional coactivators p/CIP and SRC-1 control insulin resistance through IRS1 in obesity models. *PLoS One* 7:e36961
55. Qin L, Liu Z, Chen H, et al. 2009. The steroid receptor coactivator-1 regulates Twist expression and promotes breast cancer metastasis. *Cancer Res.* 69:3819–27
56. McBryan J, Theissen SM, Byrne C, et al. 2012. Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1. *Cancer Res.* 72:548–59

57. McCartan D, Bolger JC, Fagan A, et al. 2012. Global characterization of the SRC-1 transcriptome identifies ADAM22 as an ER-independent mediator of endocrine-resistant breast cancer. *Cancer Res.* 72:220–29
58. Osborne CK, Bardou V, Hopp TA, et al. 2003. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J. Natl. Cancer Inst.* 95:353–61
59. Mann M, Cortez V, Vadlamudi R. 2013. PELP1 oncogenic functions involve CARM1 regulation. *Carcinogenesis* 34:1468–75
60. Ou CY, LaBonte MJ, Manegold PC, et al. 2011. A coactivator role of CARM1 in the dysregulation of beta-catenin activity in colorectal cancer cell growth and gene expression. *Mol. Cancer Res.* 9:660–70
61. Cai D, Shames DS, Raso MG, et al. 2010. Steroid receptor coactivator-3 expression in lung cancer and its role in the regulation of cancer cell survival and proliferation. *Cancer Res.* 70:6477–85
62. Taylor BS, Schultz N, Hieronymus H, et al. 2010. Integrative genomic profiling of human prostate cancer. *Cancer Cell.* 18:11–22
63. Agoulnik IU, Vaid A, Nakka M, et al. 2006. Androgens modulate expression of transcription intermediary factor 2, an androgen receptor coactivator whose expression level correlates with early biochemical recurrence in prostate cancer. *Cancer Res.* 66:10594–602
64. Agoulnik IU, Weigel NL. 2008. Androgen receptor coactivators and prostate cancer. *Adv. Exp. Med. Biol.* 617:245–55
65. Shiota M, Yokomizo A, Tada Y, et al. 2010. Peroxisome proliferator-activated receptor gamma coactivator-1alpha interacts with the androgen receptor (AR) and promotes prostate cancer cell growth by activating the AR. *Mol. Endocrinol.* 24:114–27
66. Carapeti M, Aguiar RC, Goldman JM, et al. 1998. A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* 91:3127–33
67. Carapeti M, Aguiar RC, Watmore AE, et al. 1999. Consistent fusion of MOZ and TIF2 in AML with inv(8) (p11q13). *Cancer Genet. Cytogenet.* 113:70–72
68. Fabris S, Mosca L, Cutrona G, et al. 2012. Chromosome 2p gain in monoclonal B-cell lymphocytosis and in early stage chronic lymphocytic leukemia. *Am. J. Hematol.* 88:24–31
69. Vazquez F, Lim JH, Chim H, et al. 2013. PGC1alpha expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. *Cancer Cell* 23:287–301
70. Dasgupta S, Srinidhi S, Vishwanatha JK. 2012. Oncogenic activation in prostate cancer progression and metastasis: molecular insights and future challenges. *J. Carcinog.* 11:4
71. Yi P, Xia W, Wu RC, et al. 2013. SRC-3 coactivator regulates cell resistance to cytotoxic stress via TRAF4-mediated p53 destabilization. *Genes Dev.* 27:274–87
72. Percharde M, Lavial F, Ng JH, et al. 2012. Ncoa3 functions as an essential Esrrb coactivator to sustain embryonic stem cell self-renewal and reprogramming. *Genes Dev.* 26:2286–98
73. Wu Z, Yang M, Liu H, et al. 2012. Role of Nuclear receptor coactivator 3 (Ncoa3) in pluripotency maintenance. *J. Biol. Chem.* 287:38295–304
74. O'Malley BW, Kumar R. 2009. Nuclear receptor coregulators in cancer biology. *Cancer Res.* 69:8217–22
75. Wang Y, Lonard DM, Yu Y, et al. 2011. Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. *Mol. Endocrinol.* 25:2041–53
76. O'Malley BW. 2010. Masters of the genome. *Nat. Rev. Mol. Cell. Biol.* 11:311

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An epigenomic approach to therapy for tamoxifen-resistant breast cancer

Qin Feng[1], Zheng Zhang[1], Martin J Shea[2], Chad J Creighton[3], Cristian Coarfa[3], Susan G Hilsenbeck[2], Rainer Lanz[1], Bin He[1, 4], Lei Wang[1], Xiaoyong Fu[1, 2, 3], Agostina Nardone[1, 2, 3], Yongcheng Song[5], James Bradner[6], Nicholas Mitsiades[3, 4], Constantine S Mitsiades[6], C Kent Osborne[1, 2, 3], Rachel Schiff[1, 2, 3], Bert W O'Malley[1, 3]

[1]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA; [2]Lester & Sue Smith Breast Center, Baylor College of Medicine, Houston, TX 77030, USA; [3]Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA; [4]Department of Medicine-Hematology & Oncology, Baylor College of Medicine, Houston, TX 77030, USA; [5]Department of Pharmacology, Baylor College of Medicine, Houston, TX 77030, USA; [6]Department of Medical Oncology, Dana-Farber Cancer Institute, Department of Medicine, Harvard Medical School, Boston, MA 02215, USA

Tamoxifen has been a frontline treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However, resistance to tamoxifen occurs in many patients. ER still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for treatment of tamoxifen-resistant (Tam-R) breast cancer. In an effort to identify novel regulators of ERα signaling, through a small-scale siRNA screen against histone methyl modifiers, we found WHSC1, a histone H3K36 methyltransferase, as a positive regulator of ERα signaling in breast cancer cells. We demonstrated that WHSC1 is recruited to the *ERα* gene by the BET protein BRD3/4, and facilitates *ERα* gene expression. The small-molecule BET protein inhibitor JQ1 potently suppressed the classic ERα signaling pathway and the growth of Tam-R breast cancer cells in culture. Using a Tam-R breast cancer xenograft mouse model, we demonstrated *in vivo* anti-breast cancer activity by JQ1 and a strong long-lasting effect of combination therapy with JQ1 and the ER degrader fulvestrant. Taken together, we provide evidence that the epigenomic proteins BRD3/4 and WHSC1 are essential regulators of estrogen receptor signaling and are novel therapeutic targets for treatment of Tam-R breast cancer.

***Keywords*:** epigenomic; tamoxifen; breast cancer

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Introduction

Estrogen signaling is crucial for the development of normal mammary gland and breast cancer. Estrogen binds to and activates estrogen receptors (ERs), resulting in expression of genes involved in cell proliferation and survival. By blocking estrogen binding to ER alpha (ERα), the selective ER modulator, tamoxifen remains a frontline treatment for ERα-positive breast cancer [1]. However, eventually many tumors develop tamoxifen resistance. Interestingly, ERα is still important for the growth of breast cancer cells with acquired drug resistance. For instance, recent chromatin immunoprecipitation (ChIP)-seq analysis on primary breast tumors from patients clearly shows that ERα is still recruited to chromatin in drug-resistant breast cancer [2]. Moreover, in the absence of estrogen, epidermal growth factor could induce AP-1-dependent ERα genomic targets [3]. Therefore, there is an urgent need to develop novel treatments to further suppress ER signaling in Tam-R breast cancer. Recent data show that mTOR inhibition is effective in overcoming hormone resistance [4]. However, mTOR inhibition is associated with several side effects, limiting its use in patients who must take it for a long time. Combinations of HDAC inhibitor vorinostat and tamoxifen also showed some effect in reversing hormone resistance [5]. In this study, we are exploring a novel strategy to overcome endocrine resistance by shutting down expres-

Correspondence: Bert W O'Malley
E-mail: berto@bcm.edu
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sion of the *ER* gene itself.

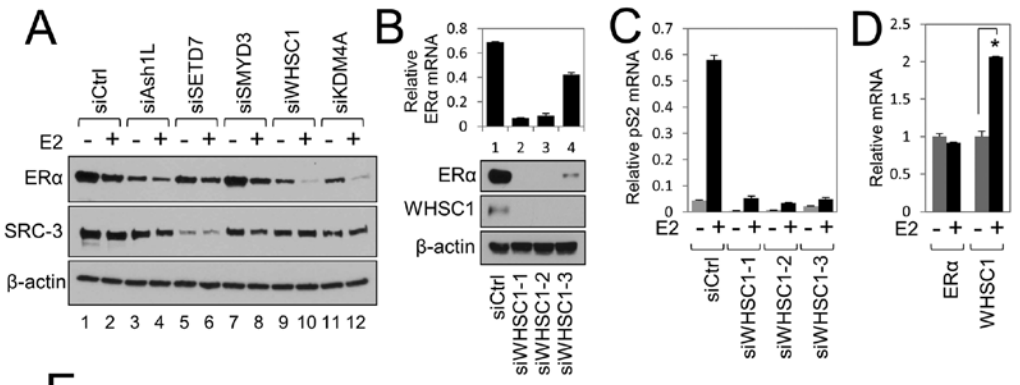
Results

WHSC1 regulates ERα gene expression

Recent progress has shown that histone-modifying enzymes and coregulators play important roles in the regulation of gene expression [6-8]. In this study, we were interested in understanding how histone modifications, particularly histone methylation, regulate ERα signaling in breast cancer cells. To this end, we performed a small-scale siRNA screen to identify epigenomic enzymes involved in estrogen signaling. We knocked down histone methyltransferases (HMTs) or demethylases (HDMTs) individually in MCF7 cells by siRNA, and determined the expression levels of ERα and its target genes *GREB1* and *pS2*. We focused on the HMTs and HDMTs that target lysine residues, because the diverse roles of histone lysine methylation in gene expression have been well established. The role of 29 HMTs and 18 HDMTs in estrogen signaling was tested by knockdown using individual siRNAs. Supplementary information, Figure S1 shows the mRNA levels of ERα and its target gene *GREB1*. Knockdown of SMYD3 led to an increased level of *GREB1* mRNA, while knockdown of three HMTs, ASH1L, SETD7 and WHSC1, and two HDMTs, KDM4A and KDM7C reduced *GREB1* mRNA level by > 60%. Similar effects were observed for *pS2* gene expression (data not shown). ERα mRNA levels were also significantly reduced in these samples, indicating that these epigenomic enzymes are critical for ERα and its target gene expression. We confirmed the reduction of ERα protein levels by western blot analysis as shown in Figure 1A.

WHSC1 encodes a HMTs that methylates histone H3K36 [9, 10]. Methylation of H3K36 is a key histone mark for transcription elongation. *WHSC1* was initially found to be deleted in Wolf-Hirschhorn syndrome (WHS), which is a malformation syndrome associated with a hemizygous deletion of the distal short arm of chromosome 4. Later studies revealed that *WHSC1* is significantly overexpressed in many cancers including breast cancer [11, 12]. Importantly, its expression is associated with tumor aggressiveness and bad prognosis in several breast cancer studies [13, 14]. However, the underlying molecular mechanism of its role in breast cancer development remains unknown. Therefore, we focused on WHSC1 for further study.

Using three individual *WHSC1* siRNAs, we confirmed the essential role of WHSC1 in expression of ERα and its target genes (Figure 1B and 1C). Interestingly, *WHSC1* mRNA levels were increased by two-fold in MCF7 cells treated with estradiol, indicating that *WHSC1* itself is an ERα-regulated gene (Figure 1D), forming a positive



E

Tumor Type	ER-positive	Her2	Luminal A	Luminal B
Pearson's r	0.245	0.037	0.285	0.125
P value	$8.72*10^{-6}$	0.4025	$3.48*10^{-5}$	0.095

Figure 1 WHSC1 regulates *ERα* gene expression. **(A)** Confirmation of siRNA screening results by western blot analysis. Five genes were selected from the siRNA screening. The protein levels of ERα and SRC-3 were measured by western blot analysis. **(B)** Knockdown of WHSC1 by three different siRNAs all decreased mRNA and protein levels of ERα in MCF7 cells. **(C)** Knockdown of *WHSC1* reduced the expression of ERα target gene *pS2* in MCF7 cells. 10 nM of estradiol (E2) was added 24 h before cell harvest. **(D)** Enhanced expression of WHSC1 by treatment of 10 nM E2 for 24 h in MCF7 cells. mRNA levels of ERα and WHSC1 were measured by RT-qPCR. *$P < 0.05$ by a two-tailed *t*-test. **(E)** Correlation of ERα mRNA and WHSC1 mRNA levels in a subset of the TCGA breast cancer samples.

feedback regulatory loop. In support of this, we found a positive correlation in mRNA levels between WHSC1 and ERα in ER-positive patients in the TCGA database, particularly in luminal-A-type of breast tumors (Figure 1E).

WHSC1 and BRD3/4 coordinately regulate ERα expression and function

Because WHSC1 does not contain a DNA-binding domain, it cannot bind directly to the ERα gene promoter/enhancer to regulate its transcription. To investigate how WHSC1 activates ERα gene expression, we searched the Epicome database (http://epicome.org), a mass spectrometry-based proteomics database generated by the Nuclear Receptor Signaling Atlas (NURSA) [15, 16], to identify WHSC1-interacting proteins. BRD3 and BRD4, two bromodomain-containing proteins, are at the top of a list of potential WHSC1-interacting proteins (Supplementary information, Figure S2A). Their association with WHSC1 was confirmed by immunoprecipitation/western blot analysis using either exogenously expressed or endogenous proteins. As shown in Figure 2A-2C, BRD3 and BRD4 were co-immunoprecipitated with WHSC1 protein and vice versa, but another BET family protein, BRD2, did not interact significantly with WHSC1. Similar interaction was detected between endogenous BRDs and WHSC1 in MCF7 cells (Supplementary information, Figure S2), and this is in agreement with a recent report of an interaction between WHSC1 and BRD4 in MEF cells [17].

BRD3 and BRD4 belong to the BET (bromodomain and extraterminal domain) family of proteins. In humans, there are four BET proteins including BRD2, BRD3, BRD4, and BRDT [18]. BET family members contain two bromodomain and one extraterminal domain. The bromodomain specifically recognizes acetylated lysine residues on the histone tails. BRD3 and BRD4 are implicated in the transcription elongation process by association with the PAF1 complex and the pTEFb complex, respectively [19]. BRD4 also regulates the expression of G1 cell cycle genes [20]. We next determined the domain responsible for the interaction between WHSC1 and BRD3/4 by deletion mapping. As shown in Supplementary information, Figure S2D and S2E, BRD4 interacts with WHSC1 through its N-terminal 470 amino acids, which contains two bromodomains. Interestingly, treatment of cells with JQ1 failed to disrupt the interaction between WHSC1 and BRD4, suggesting that BRD4 can bind to WHSC1 and acetylated lysine simultaneously (Supplementary information, Figure S2F).

Given the key role of WHSC1 in ERα gene expression and the physical association between WHSC1 and BRD3/4, we hypothesized that WHSC1 is recruited to the ERα gene promoter by BRD3/4 which binds directly to acetylated histone tails and subsequent methylation of K36 on histone H3 by WHSC1, then could facilitate the transcription elongation of the *ERα* gene. To test this hypothesis, we asked whether BRD3/4 are required for the expression of ERα and its target genes. As shown in Figure 2D, simultaneous knockdown of BRD3 and BRD4 dramatically reduced the mRNA levels of ERα and *pS2*, indicating that BRD3 and BRD4 are crucial for the expression of ERα.

To further test the above hypothesis, BRD3 and BRD4 genes were knocked down in MCF7 cells, and the recruitment of WHSC1 to the *ERα* gene and the level of histone H3 K36 methylation were determined by ChIP-qPCR assay. As shown in Figure 2E, knockdown of BRD3/4 dramatically reduced the recruitment of WHSC1 to the *ERα* gene and the levels of H3K36me2 and H3K36me3 were both significantly reduced, suggesting that BRD3/4 functions as a scaffold to recruit WHSC1, which promotes the transcription elongation of the *ERα/ESR1* gene.

In our model shown in Figure 2F, BET proteins (BRD3/4) recognize acetylated lysine residues on histone tails in the *ERα/ESR1* gene promoter. After recruitment to the *ERα* gene by interacting with BRD3/4, WHSC1 methylates H3K36 and subsequently increases transcription elongation of ERα. On the other hand, estrogen stimulates the expression of WHSC1, forming a positive feedback regulatory loop. Interestingly, we also found that WHSC1 and BRD4 are potent coactivators for ER in an ERE-luciferase gene reporter assay (Supplementary information, Figure S3A). Taken together, our results indicate that WHSC1 is a key regulator of ER signaling, as it is not only a positive epigenomic regulator of ER gene expression, but also a coactivator for ER itself.

Small-molecule inhibitors have been recently developed and published for the BET family of proteins. For instance, JQ1, an acetylated lysine analog, has been reported in recent studies to be a potent BET inhibitor and can be used to treat a number of cancers including multiple myeloma and acute myeloid leukemia [21-23]. Based on our results, we tested whether JQ1 can suppress ERα expression. As shown in Figure 2G, treatment of MCF7 cells with JQ1 significantly reduced the mRNA levels of ERα and its target genes *pS2, GREB1*, and *Cyclin D1*, but not other breast cancer genes such as *FoxA1, SRC-3*, and *Her2* (Supplementary information, Figure S3B). Another bromodomain inhibitor I-BET had a similar effect, although at a slightly higher concentration (Figure 2H). Similar experiments were performed on prostate cancer LNCaP cells. Interestingly, androgen receptor mRNA

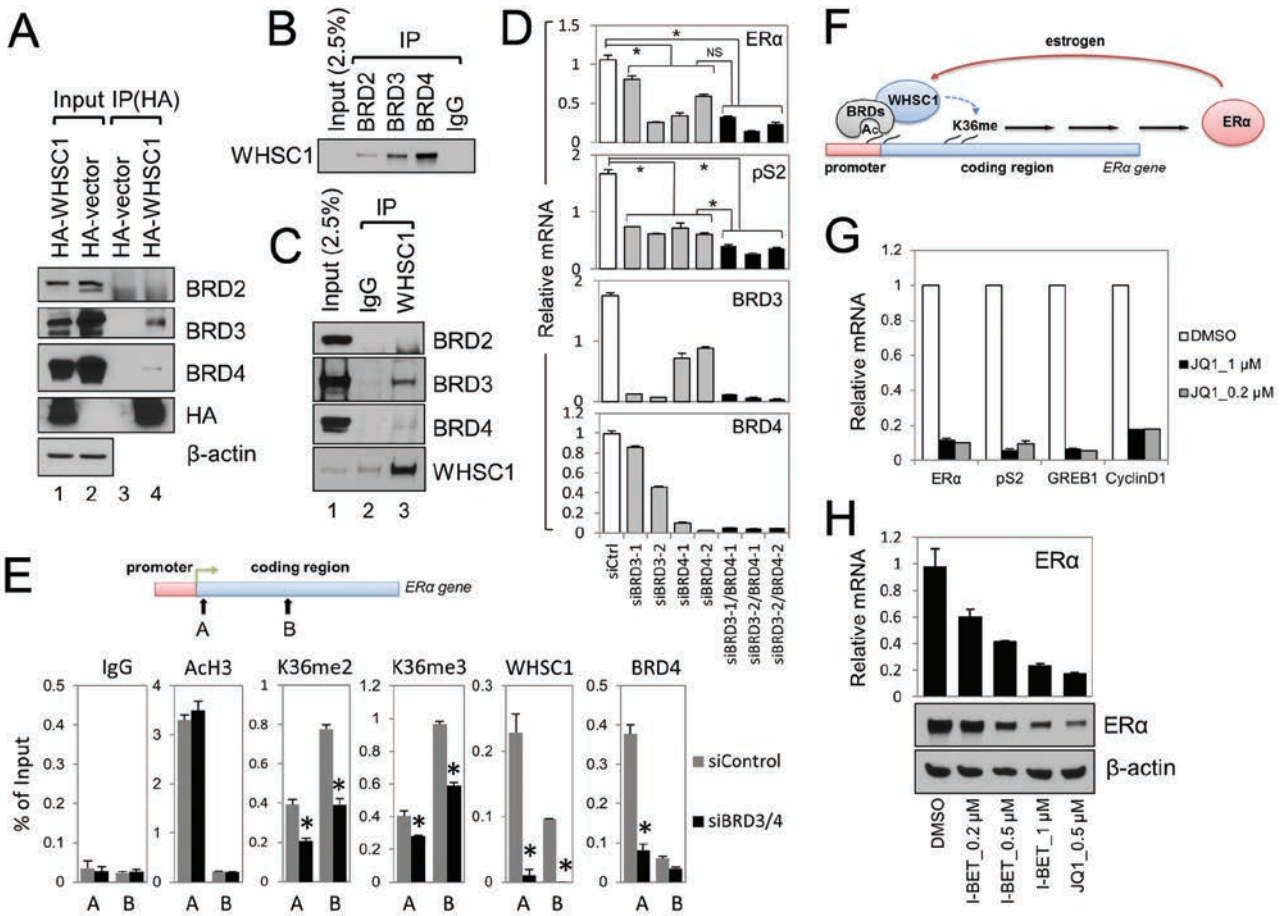


Figure 2 WHSC1 and BRD3/4 coordinately regulate ERα expression and function. **(A)** Co-immunoprecipitation (co-IP) of WHSC1 and BRD proteins from transiently transfected 293T cells. HA-tagged WHSC1 or empty vector was transiently transfected to 293T cells for 48 h. The whole-cell lysate was cleared and immunoprecipitated with anti-HA antibody. Input, 2.5%. **(B)** Co-IP of endogenous WHSC1 and BRD proteins from HeLa nuclear extract. Input, 2.5%. **(C)** Reciprocal co-IP of endogenous WHSC1 and BRD proteins from HeLa nuclear extract. Input, 2.5%. **(D)** Knockdown of BRD3 and BRD4 reduced expression of ERα and its target gene *pS2* in MCF7 cells. Error bars indicate SEM. **P* < 0.01 by *t*-test. **(E)** Loss of BRD3/4 abolished the recruitment of WHSC1 to *ERα* gene. ChIP was performed in MCF7 cells treated with BRD3/4 siRNA or control siRNA for 2 days. Primer pair A locates next to promoter region and primer pair B locates in the coding region of ERα. Each IP was duplicated and average values were shown. Error bars indicate SEM. **P* < 0.05 by *t*-test. **(F)** A hypothetical model of regulation of ERα gene expression by WHSC1 and BRD3/4. **(G)** Bromodomain inhibitor JQ1 efficiently reduced the expression of ERα and its target genes in MCF7 cells. Cells were treated with JQ1 for 24 h before harvest. **(H)** Another Bromodomain inhibitor I-BET had similar function in suppressing ERα expression. MCF7 cells were treated with I-BET for 24 h before harvest.

levels were not altered upon JQ1 treatment, indicating that the suppression of ERα expression by JQ1 is selective (Supplementary information, Figure S3C).

JQ1 inhibits growth of Tam-R breast cancer cells

Next, we investigated whether the BRD3/4 inhibitor JQ1 inhibits growth of breast cancer cells. We treated parental MCF7 cells and a Tam-R derivative with different doses of JQ1 or vehicle control. As shown in Figure 3A, JQ1 potently inhibited the growth of Tam-R MCF7 cells, while it only moderately inhibited the growth of parental MCF7 cells at a concentration of 0.2 μM (At a higher concentration of 0.5 μM, JQ1 also inhibited parental MCF7 cell growth.). We further tested three more ER-positive breast cancer cell lines including T47D, MCF7 RN, and ZR75-1 cells. We found that JQ1 inhibited the growth of all of these breast cancer cell lines, with higher efficacy toward the Tam-R cells. Interestingly, JQ1 also inhibited four estrogen-deprivation-resistant (mimics aromatase inhibitor resistance) lines (Supplementary information, Figure S4A). Simlar to MCF7 cells, JQ1 downregulated ERα mRNA levels in MCF7

RN and ZR75-1 cells. The ERα dominant-negative splicing variant ERΔ7 was similarly downregulated [24] (Supplementary information, Figure S4B and S4C).

Our ChIP-qPCR analysis provided further evidence that disruption of BRD3/4/WHSC1/ERα axis by JQ1 suppressed ERα gene expression. As shown in Figure 3B, overall, the levels of histone modifications marking active promoters, such as acetylated histone H3 and H3K4me3, were significantly lower on the ERα gene promoter in Tam-R MCF7 cells, likely due to lack of other key transcriptional factor(s). JQ1 treatment in Tam-R cells eliminated the recruitment of BRD3/4 and WHSC1 to the ERα gene promoter, and dramatically reduced the level of H3K4me3 and H3K36me3, two histone marks of transcription activation. In parental MCF7 cells, the recruitment of BRD3/4 and WHSC1 was still maintained at relatively high levels, although it was reduced by JQ1. BRD3 and BRD4 mRNA levels were not reduced (actually increased) by JQ1, while WHSC1 was reduced by JQ1 both at the mRNA and protein levels (Figure 3C and 3D).

We next wished to understand why Tam-R breast cancer cells are more sensitive to JQ1. Knockdown of WHSC1 alone reduced the ERα mRNA levels similarly in both parental and Tam-R breast cancer cells, and inhibited the growth of parental and Tam-R MCF7 cells similarly (Supplementary information, Figure S4D and S4E). This result suggests that WHSC1 is an important regulator of ERα gene expression and cell growth, but does not cause extra JQ1 sensitivity in Tam-R cells. However, we noticed that when cells were treated with JQ1 for up to 3 days, ERα mRNA was persistantly suppressed in Tam-R MCF7 cells (Figure 4A). In contrast, in parental MCF7 cells, ERα mRNA level was abolished initially, but recovered after prolonged treatment (Figure 4A). Moreover, JQ1 was reported to inhibit MYC signaling in previous studies [21, 25, 26]. Thus, we measured expression of MYC in JQ1-treated MCF7 cells. As shown in Figure 4A, MYC mRNA level responded to JQ1 treatment similarly to ER in parental and Tam-R MCF7 cells. These results demonstrate that ERα and MYC are JQ1 target genes in Tam-R MCF7 cells, and that sustained sup-

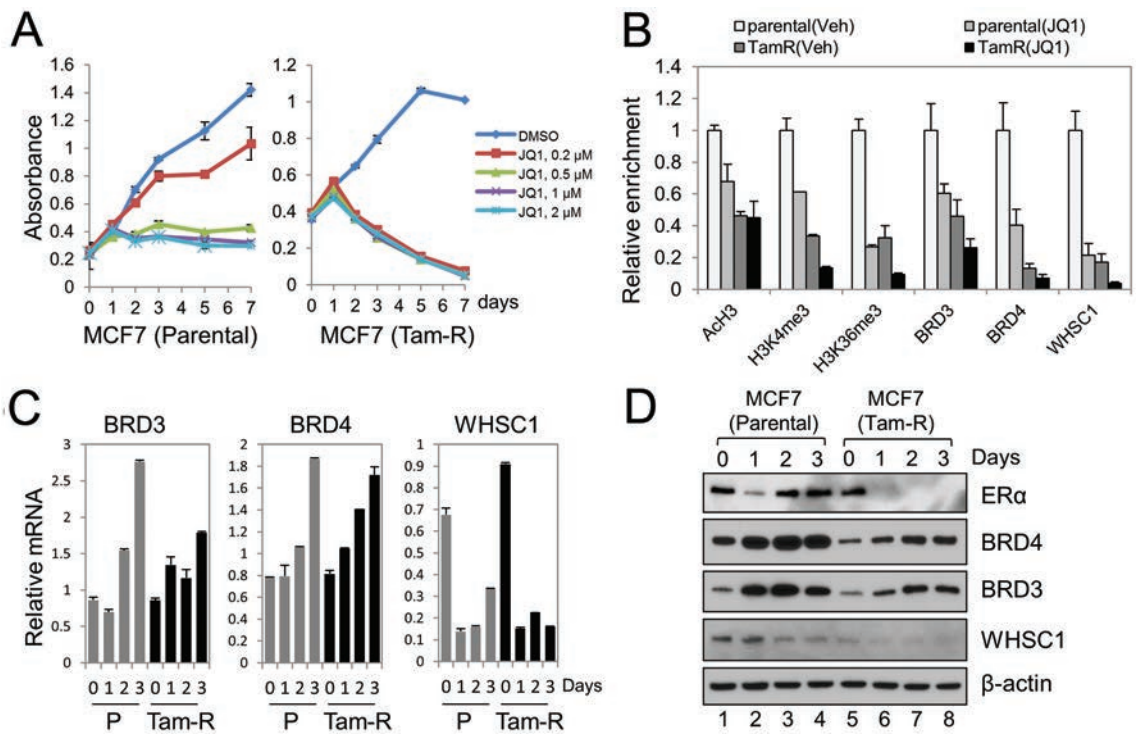


Figure 3 JQ1 inhibits growth of Tam-R cells. **(A)** JQ1 efficiently inhibited the growth of Tam-R MCF7 breast cancer cells as determined by MTS assay. Error bars indicate SEM. **(B)** ChIP assay to determine the level of histone modifications and recruitment of BRD3/4 and WHSC1 to ERα promoter. MCF7 parental and Tam-R cells were treated with DMSO (Veh) or 0.2 M of JQ1 for 24 h before cell harvest. Primer Pair A (shown in Figure 2E) was used for qPCR analysis. Error bars were shown as SEM. **(C)** Expression of BRDs and WHSC1 in 0.2 μM JQ1-treated MCF7 cells. **(D)** Western blot analysis of ERα, BRDs, and WHSC1 proteins from 0.2 μM JQ1-treated MCF7 cells.

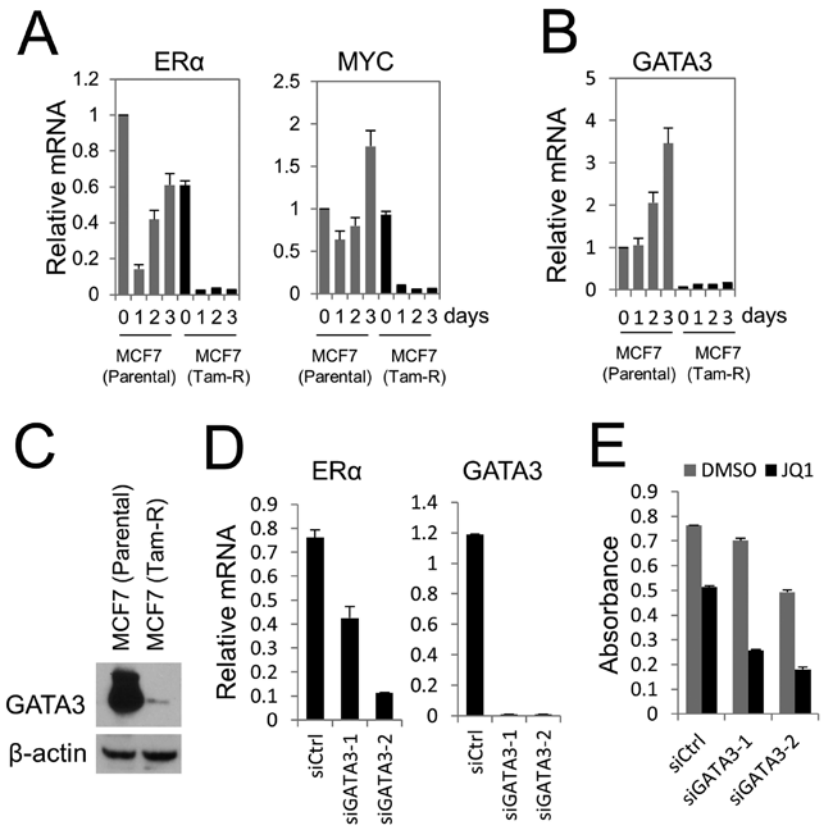
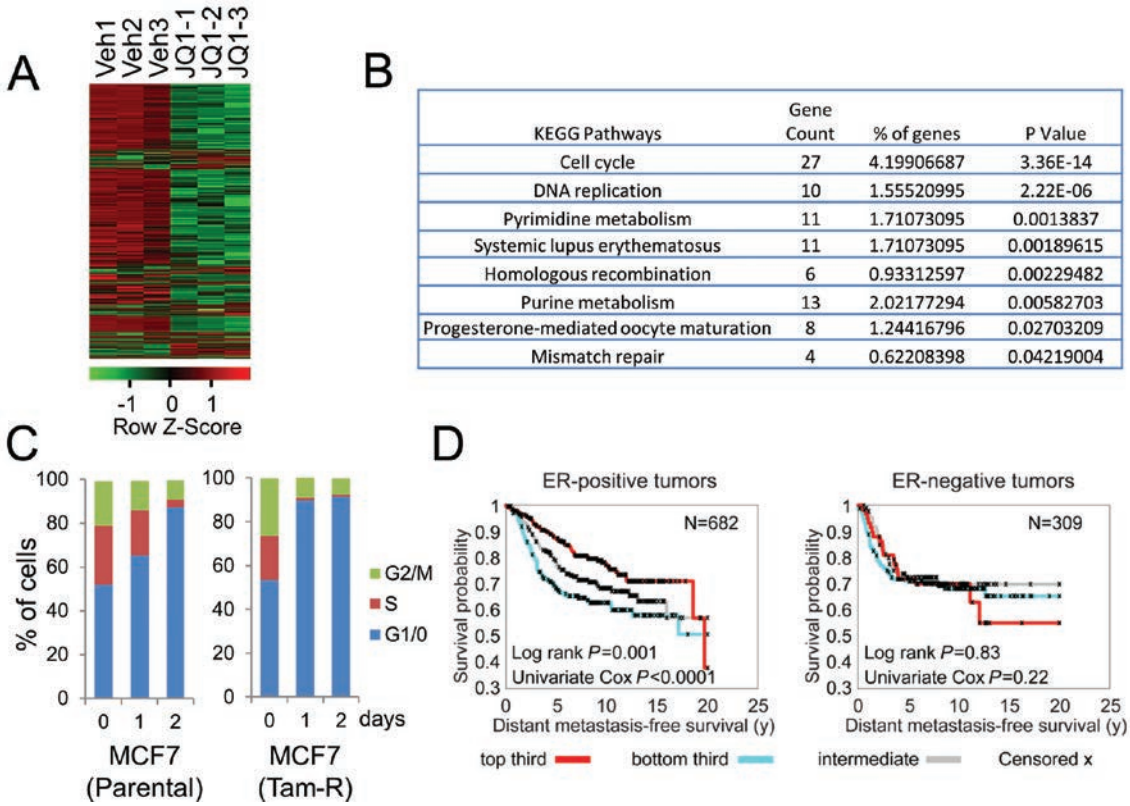


Figure 4 GATA3 is a potential factor to regulate JQ1 sensitivity in MCF7 cells. **(A)** JQ1 suppressed both ERα and MYC signaling pathways in Tam-R MCF7 cells. Parental and Tam-R MCF7 cells were treated with 0.2 μM of JQ1 for different days, and mRNA levels of ERα and MYC were analyzed by RT-qPCR. **(B)** Expression of GATA3 in MCF7 parental and Tam-R cells after JQ1 treatment. **(C)** Comparison of GATA3 protein levels by western blot analysis in MCF7 parental and Tam-R cells. **(D)** Knockdown of GATA3 by siRNA reduces ERα gene expression. **(E)** Knockdown of GATA3 by siRNA enhances JQ1 inhibition function in MCF7 parental cells. All error bars were shown as SEM.

pression of ERα and MYC by JQ1 probably contributes to its more potent anticancer activity on Tam-R breast cancer cells. To gain more mechanistic insight into this observation, we found that GATA3, a key regulator of ER gene expression [27], is highly expressed in parental MCF7 cells, but not in Tam-R cells (Figure 4B and 4C). In parental MCF7 cells, GATA3 expression is further increased by JQ1 treatment (Figure 4B). When we knocked down GATA3 using siRNA, the parental MCF7 cells became more sensitive to JQ1 treatment (Figure 4E). Thus, our results suggest that other key transcription factors, such as GATA3 in parental MCF7 cells, could have contributed to the JQ1 resistance with prolonged treatment. A decrease in such factors (Figure 4B) might contribute to epigenomic environmental changes on the ERα promoter, resulting in greater JQ1 sensitivity in Tam-R lines.

To determine the global signaling pathways that are altered by JQ1 in addition to ER and MYC, microarray analysis was performed on Tam-R MCF7 cells treated with vehicle or 0.2 μM of JQ1. When applying a threshold of $\log2 < -0.2$ or $\log2 > 0.2$, we identified 652 downregulated genes and 219 upregulated genes in JQ1-treated cells (Figure 5A). Supplementary information, Table S1A lists all the genes upregulated or downregulated in major biological pathways by KEGG pathway analysis. Figure 5B shows the biological pathways negatively affected by JQ1. Among them, the cell cycle is an important pathway being affected since cell cycle-related gene expression was significantly altered by JQ1 treatment (Supplementary information, Figure S5A). Consistent with this observation, we found by flow cytometry analysis that Tam-R MCF7 cells were arrested in G1 phase after JQ1 treatment for 24 h, while parental cells were arrested in G1 phase after > 48 h of JQ1 treatment (Figure 5C). Moreover, using these JQ1 target genes, we generated a JQ1-regulated gene signature using the same method as previously described [28]. Using a compendium of several expression array studies, we scored human breast tumors based on the manifestation of the JQ1 gene signature. As shown in Figure 5D, in ER-positive tumors (N = 682), high JQ1 signature activity was associated with better patient outcome (log-rank P = 0.001), while in ER-negative tumors (N = 309), no survival association was found. These data further support the functional significance of JQ1 in ER signaling in breast cancer.

After 2 days of JQ1 treatment, Tam-R cells began to die, suggesting that prolonged cell cycle arrest may induce apoptosis (Figure 3A). This was confirmed by the appearance of cleaved PARP-1 protein in Tam-R MCF7 cells (Supplementary information, Figure S5B). In con-



KEGG Pathways	Gene Count	% of genes	P Value
Cell cycle	27	4.19906687	3.36E-14
DNA replication	10	1.55520995	2.22E-06
Pyrimidine metabolism	11	1.71073095	0.0013837
Systemic lupus erythematosus	11	1.71073095	0.00189615
Homologous recombination	6	0.93312597	0.00229482
Purine metabolism	13	2.02177294	0.00582703
Progesterone-mediated oocyte maturation	8	1.24416796	0.02703209
Mismatch repair	4	0.62208398	0.04219004

Figure 5 Cellular pathways targeted by JQ1. **(A)** Heatmap of expression levels for the genes differentially expressed upon treatment with JQ1. Tam-R MCF7 cells were treated with 0.2 μM of JQ1 or vehicle (DMSO) for 24 h before harvest for microarray analysis. **(B)** Biological pathways were identified by microarray analysis. KEGG pathways were determined by the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) based on the gene list that is downregulated by JQ1. **(C)** Tam-R MCF7 cells are more sensitive to JQ1-induced G1 cell cycle arrest. Cells were fixed and stained with propidium iodide (PI) before being analyzed by flow cytometry. **(D)** Association of the gene expression signature of JQ1 treatment with breast cancer patient survival. For ER-positive and ER-negative subsets, the differences in risk between tumors, according to degree of manifestation of the JQ1 gene signature, is compared using Kaplan-Meier plots (top third, "strong manifestation"; bottom third, "weak manifestation"; middle third, "intermediate manifestation").

trast, parental MCF7 cells did not undergo apoptosis (Supplementary information, Figure S5B).

JQ1 inhibits tumor growth in Tam-R xenograft mouse model when combined with fulvestrant therapy

Next, we sought to determine the *in vivo* antitumor activity of JQ1. Ovariectomized nude mice were transplanted subcutaneously with Tam-R MCF7 tumors, and were randomized into two groups when tumor volumes reached 200 mm^3. JQ1 or control vehicle was given to these mice by intraperitoneal injection daily. We first performed a pilot experiment to treat the mice for 7 days to test whether JQ1 could downregulate ERα expression *in vivo*. As shown in Supplementary information, Figure S6A, the ERα mRNA level was indeed reduced by about 30% in JQ1-treated tumors; MYC mRNA level was not significantly reduced, although there was a trend. Immunohistochemical staining confirmed a decreased level of ERα protein in JQ1-treated tumors, and a reduced proliferation rate was observed with Ki67 and histone H3 phospho-Ser10 staining (Supplementary information, Figure S6B). This result demonstrates that JQ1 has *in vivo* anticancer activity against Tam-R breast cancer. To achieve an optimal drug response *in vivo*, we tested a combination of JQ1 and fulvestrant/ICI 182,780, an ERα protein degrader, in Tam-R MCF7 xenograft tumors. While single treatment of JQ1 or fulvestrant moderately inhibited tumor growth, the combination of JQ1 and fulvestrant showed a synergistic antitumor activity in the Tam-R tumors. In the vehicle-treated group, the volumes of all the tumors quickly tripled within 17 days, while in the group that received combination therapy, none of the tumors tripled their size after 40 days of treatment, and only about half of the tumors tripled after a prolonged

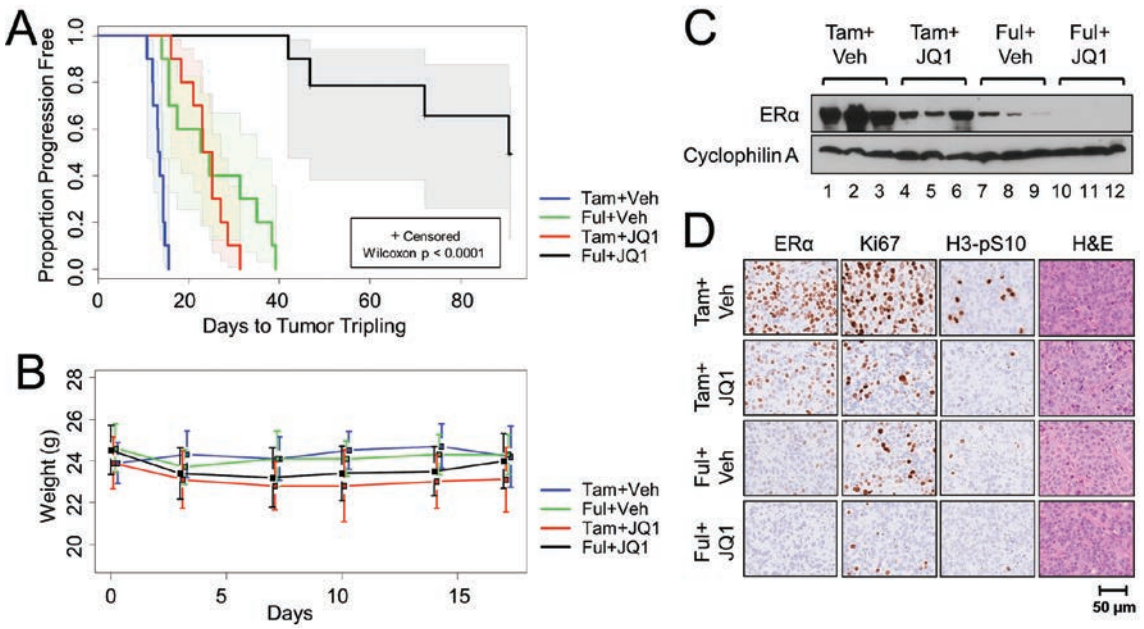


Figure 6 A combination therapy of JQ1 and fulvestrant in Tam-R xenograft mouse model. **(A)** Ovariectomized mice bearing Tam-R-established MCF7 tumors were randomized (on day 0) into four groups of treatment: Tam+vehicle, Tam+JQ1, fulvestrant+vehicle, and fulvestrant+JQ1, with 10 mice per group. JQ1 was administered daily at 50 mg/kg, while 5 mg fulvestrant was given by subcutaneous injection weekly. Tumors were harvested when they reached 1 000 mm^3 or 3 months after treatment. **(B)** Body weight measurement for xenograft experiment shown in **A**. The error bars show means ± SEM. **(C)** Tumors were harvested by the end of the treatment, and western blot was performed using antibodies against ERα and cyclophilin A. **(D)** Immunohistochemical staining of ERα, Ki67, and histone H3 phospho-Ser10 in xenograft Tam-R tumors from four groups treated with Tam+vehicle, Tam+JQ1, fulvestrant+vehicle, or fulvestrant+JQ1.

treatment (90 days) (Figure 6A). Time-to-tumor tripling from the four groups of treatment was compared using the generalized Wilcoxon test as shown in Supplementary information, Figure S6C. Although JQ1-treated animals lose weight initially and then regain it, generally there is no difference among the four groups in the baseline weights, indicating that JQ1 is well tolerated by mice (Figure 6B). By western blot analysis, the protein levels of ERα were dramatically downregulated in the tumors that received combination therapy of fulvestrant and JQ1 (Figure 6C). Immunohistochemical staining for Ki67 and histone H3 phospho-Ser10 confirmed that the combination therapy potently inhibited the tumor cell proliferation (Figure 6D).

Discussion

Epigenetics is defined as heritable changes caused by mechanisms other than changes in DNA sequence, including DNA methylation, histone modifications, and noncoding RNA. Epigenomic proteins such as chromatin regulators have emerged as novel therapeutic targets for cancer. For instance, DNA methylase inhibitors, such as Vidaza and Decitabine, and an HDAC inhibitor, such as Vorinostat, have been used clinically in treating hematological malignancies [29]. Specific inhibitors against H3K79 methylase hDOT1L and H3K27 methylase EZH2 also are being developed for treatment of a variety of cancers [30-32]. The small-molecule inhibitor of epigenomic reader bromodomain JQ1 has shown potent anticancer activity in hematological cancer by targeting the MYC pathway. JQ1 is also thought to be effective in a subset of human lung adenocarcinoma cell lines by inhibiting the expression of an oncogenic transcriptional factor FOSL1 [33]. In a previous report, breast cancer cell lines only show modest sensitivity to JQ1 [34]. In agreement with that observation, we found that the growth of the parental MCF7 and T47D cells was only partially inhibited by JQ1 at high concentrations. Strikingly, the Tam-R cells are more sensitive to JQ1 treatment. In our JQ1 treatment experiment, the ER mRNA level in MCF7 parental cells initially was suppressed significantly but returned to a normal level after prolonged treatment. MYC mRNA levels were only slightly changed by JQ1 in MCF7L parental cells. In contrast, in Tam-R cells, both ER and MYC mRNA levels were consistently sup-

pressed by JQ1 even after 3 days of treatment. Our result provides an explanation for the increased JQ1 sensitivity of Tam-R breast cancer cells compared to parental cells, and further indicates that JQ1 targets both ER ad MYC pathways in Tam-R cells. More importantly, JQ1 shows *in vivo* anticancer activity in suppressing the Tam-R breast cancer growth in the xenograft mouse model. A combination treatment of fulvestrant and JQ1 more effectively downregulated ERα and inhibited *in vitro* and *in vivo* tumor growth, providing a new potential approach for treating Tam-R and ERα-dependent breast cancer.

Materials and Methods

Small-scale siRNA screening

A customized small siRNA library contains individual Stealth RNAi siRNAs (Life Technologies) targeting 29 histone lysine methyltransferases and 18 histone lysine demethylases. All siRNAs used in this study were transfected to MCF7 cells at the final concentration of 20 nM using Lipofectamin RNAiMAX reagent.

Cell culture and transfection

MCF7, MCF7 RN, T47D, and ZR75-1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. MCF7 cells were initially obtained from Dr Marc Lippman in 1985 at the National Cancer Institute, Bethesda, MD. T47D cells were purchased from ATCC. MCF7 RN cells were initially obtained from Dr Robert Nicholson [35]. The Tam-R lines were established from a long-time treatment of 100 nM 4-hydroxy tamoxifen (4-HT) (Sigma) until cell growth was resumed. The parental cells were cultured in RPMI1640 supplemented with 10% of FCS, whereas their Tam-R derivative line was cultured in phenol red-free medium containing 10% charcoal-dextran-stripped FCS and 100 nM of 4-HT. All cell lines were authenticated once the resistance was established, and mycoplasma contamination is tested once every 6 months. For estradiol-induced experiments, MCF7 cells were maintained in phenol red-free medium containing 10% charcoal-dextran-stripped FCS until hormone addition. Fugene 6 transfection reagent (Roche) was used for transient overexpression experiments. All siRNAs used in this study were individual Stealth RNAi siRNAs from Life Technologies, and transfected at the final concentration of 20 nM. Lipofectamin RNAiMAX reagent was used for all the siRNA transfections. The mammalian expression vector of WHSC1/MMSET (pCEFL-MMSET-II) and its control vector plasmids were kindly provided by Dr Zhenkun Lou at Mayo Clinic [36]. The six different mammalian expression vectors of BRD4 used for deletion mapping in Supplementary information, Figure S2D were obtained from Addgene.

Co-IP and western blot analysis

Co-IP experiments were done to determine the interaction between WHSC1 and BRD proteins. Two days after transient transfection, 293T cells were harvested and washed with ice-cold phosphate-buffered saline before being disrupted with lysis buffer (50 mM Tris, 100 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, 0.5 μg/ml of leupeptin, and 0.7 μg/ml of pepstatin). 0.8 mg of protein lysates was incubated with 5 μl of anti-HA antibody (Roche) at 4 °C for 4 h, followed by the addition of 10 μl of protein G slurry (Santa Cruz Biotechnology) for 1 h. Endogenous IP and co-IP were performed using nuclear extract generated from HeLa cells as previously described [37]. After three washes with lysis buffer, the immunoprecipitated proteins were separated by SDS-PAGE and analyzed by western blot. The antibodies used in co-IP and western blot are anti-BRD2 (Bethyl Labs, A302-583A), anti-BRD3 (Bethyl Labs, A302-368A), anti-BRD4 (Bethyl Labs, A301-985A50), anti-WHSC1 (Abcam, ab75359), anti-β-actin (Sigma, A2228), anti-ERα (Santa Cruz Biotechnology, sc-543), and anti-cyclophilin A (Cell Signaling Technology, 2175S). The specificity of the BRD antibodies was confirmed by IP/western blot analysis (Supplementary information, Figure S2B). The validation profiles of all the antibodies are available from their company websites.

Chromatin IP

The ChIP-IT Express kit (Active Motif) was used for ChIP assay in this study following the manufacturer's protocol. The additional antibodies used in ChIP assay were rabbit IgG (Santa Cruz Biotechnology, sc-2027), anti-acetyl-histone H3 (Millipore, 06-599), anti-H3K36me2 (Active Motif, 39255), anti-H3K4me3 (Active Motif, 39159), and anti-H3K36me3 (Active Motif, 61101). These antibodies are all ChIP grade and have been validated by their companies. The ChIP PCR primers for amplification of *ERα/ESR1* gene next to the promoter region (A) are: Forward, 5′-CCCACTCAACAGCGTGTCT-3′; Reverse, 5′-CTGCAGGAAAGGCGACAG-3′. The ChIP primers for amplification of *ERα* gene in the coding region (B) are: Forward, 5′-GAAGAAGCATGGGTAAATGTCA-3′; Reverse, 5′-TCAGCCCTGAACCCAGTG-3′.

RNA isolation and reverse transcription-qPCR analysis

Total RNA was extracted with TriReagent (Molecular Research Center) (for siRNA screening) or RNeasy Mini Kit (Qiagen). To measure the relative mRNA levels, real-time reverse transcription-PCR (RT-PCR) was performed in an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The primers for gene expression assays were designed using online Roche website: https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp. The primers for amplication of total *ERα*: Forward, 5′-ATCCACCTGATGGCCAAG-3′; Reverse, 5′-GCTCCATGCCTTTGTTACTCA-3′. The primers for amplification of specifically *ERΔ7*: Forward, 5′-TGCTGGCTACATCATCTCGGTT-3′; Reverse, 5′-CCATGCCTTTGTTACAGAATTAAGCA-3′ [24]. The SensiFast SYBR one-step Kit (Bioline) was used for RT-qPCR analysis. For all RT-qPCR experiments in this study, samples were duplicated and the error bars were shown as SEM.

Correlation analysis and microarray analysis

For the correlation analysis shown in Figure 1E, gene expression and clinical data were downloaded from the TCGA Breast Cancer [38]. Pearson correlation between WHSC1 and ESR1 was computed using the *R*-statistical system within the following subtypes of breast cancer: Luminal A, Luminal B, Her2, and ER positive. For microarray analysis, the RNA was extracted with RNeasy Mini Kit (Qiagen), and the array was performed on GeneChip Human Gene 1.0 ST Array (Affymetrix) at Asuragen Inc. (Austin,

TX) with triplicated samples. A heatmap was built using the gene expression for the genes differentially expressed upon treatment with JQ1 when a threshold of log2 < −0.2 or log2 > 0.2 was applied. Gene expression was transformed by subtracting the mean value and dividing by SD for each individual gene. A heatmap was generated using the *R*-statistical system. Raw microarray data can be accessed at Gene Expression Omnibus (GEO), with accession number GSE49124.

JQ1 signature analysis

For the "compendium" data set of nine separate breast tumor expression profiling data sets for survival analysis, gene transcription profiling data sets (all on Affymetrix U133 array, A set, and all with DMFS as an outcome measure) were previously obtained from previous studies and consolidated into one data set [39]. Genes within each data set were first normalized to SD from the median, where multiple human array probe sets referenced the same gene, the probe set with the highest variation was used to represent the gene. To score each human breast tumor profile, for similarity to the gene signature of JQ1-treated cells, we derived a "*t*-score" metric for each human tumor in relation to the experimental signature, similar to what we have done in previous analyses [28, 40]; briefly, the *t*-score was defined for each external profile as the two-sided *t*-statistic comparing, within the profile, the average of the genes high in the signature with the average of the genes low in the signature.

MTS assay

Breast cancer cells were seeded at a density of 2×10^3 cells per well in flat-bottomed 96-well plates (day 0) and their growth was measured on days 1, 2, 3, 5, and 7 after JQ1 treatment. Cell media were changed every 2 days. CellTiter 96 AQueous One Solution Reagent (Promega) was added to each well following the manufacturer's instructions. After 1 h of incubation, the cell viability was determined by measuring the absorbance at 490 nm using the Multiskan FC microplate photometer (Thermo Scientific). For all the MTS assays done in this study, samples were treated in quadruplicate and error bars were shown as SEM.

Apoptosis assay

Parental and Tam-R MCF7 cells were treated with various dosages (0.2, 0.5, 1 μM) of JQ1 for 2 days followed by being harvested for western blot. The cleaved (89 kDa, C terminus) and full-length (116 kDa) forms of PARP-1 protein were detected by PARP-1 antibody (Santa Cruz Biotechnology, sc-8007, dilution 1:200).

In vivo Tam-R breast cancer xenograft studies and immunohistochemistry

The antitumor effect of JQ1 and JQ1/fulvestrant was evaluated in Tam-R breast cancer xenograft mouse model. Briefly, tamoxifen citrate-treated 4-5-week-old ovariectomized athymic mice were subcutaneously implanted with Tam-R breast tumor fragments at the hypogastrium area. Three-five weeks later when the tumor size reaches 150-200 mm^3, mice were randomized into four treatment groups by simple randomization method, totally 10 mice per group. The allocation started from the first to the fourth group, and then from the fourth to the first group for the next round. Unless any animals die from an unknown reason, no animal has been excluded from the study. JQ1 or DMSO (vehicle) was administered daily at 50 mg/kg with 10% hydroxypropyl beta-cyclodextrin solution as a carrier, while 5 mg fulvestrant was given by subcutaneous injection weekly [41]. Tumors were monitored and tumor volumes and body weight were measured twice a week. Blind measurements were carried out to avoid unconscious biases. Tumors were harvested for molecular studies after 3 months of treatment or when they reached the size of 1 000 mm^3. Small pieces of the tumors were fixed and embedded in paraffin, and additional materials were kept at –80 °C. H&E staining was performed to examine the overall structure of the tumors, and the cell proliferation was determined by immunohistochemical staining of Ki67 and phosphorylated histone H3 at Ser10. The antibodies used were histone H3 phospho-Ser10 (Millipore 06-570, 1:300), Ki67 (Dako M7240, 1:200), and ERα (Vector Laboratories VP-E613, 1:200). Other tumor parts were used for RNA extraction and protein preparation. All *in vivo* animal studies were conducted under a protocol approved by Institutional Animal Care and Use Committees (IACUC) at the Baylor College of Medicine.

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References

1 Clarke R, Liu MC, Bouker KB, *et al.* Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene* 2003; **22**:7316-7339.

2 Ross-Innes CS, Stark R, Teschendorff AE, *et al.* Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature* 2012; **481**:389-393.

3 Lupien M, Meyer CA, Bailey ST, *et al.* Growth factor stimulation induces a distinct ER(alpha) cistrome underlying breast cancer endocrine resistance. *Genes Dev* 2010; **24**:2219-2227.

4 Mayer I. Role of mTOR inhibition in preventing resistance and restoring sensitivity to hormone-targeted and HER2-targeted therapies in breast cancer. *Clin Adv Hematol Oncol* 2013; **11**:217-224.

5 Munster PN, Thurn KT, Thomas S, *et al.* A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. *Br J Cancer* 2011; **104**:1828-1835.

6 Osborne CK, Bardou V, Hopp TA, *et al.* Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003; **95**:353-361.

7 Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* 2011; **62**:233-247.

8 Huang Y, Nayak S, Jankowitz R, Davidson NE, Oesterreich S. Epigenetics in breast cancer: what's new? *Breast Cancer Res* 2011; **13**:225.
9 Kuo AJ, Cheung P, Chen K, *et al.* NSD2 links dimethylation of histone H3 at lysine 36 to oncogenic programming. *Mol Cell* 2011; **44**:609-620.
10 Nimura K, Ura K, Shiratori H, *et al.* A histone H3 lysine 36 trimethyltransferase links Nkx2-5 to Wolf-Hirschhorn syndrome. *Nature* 2009; **460**:287-291.
11 Hudlebusch HR, Santoni-Rugiu E, Simon R, *et al.* The histone methyltransferase and putative oncoprotein MMSET is overexpressed in a large variety of human tumors. *Clin Cancer Res* 2011; **17**:2919-2933.
12 Kassambara A, Klein B, Moreaux J. MMSET is overexpressed in cancers: link with tumor aggressiveness. *Biochem Biophys Res Commun* 2009; **379**:840-845.
13 Ivshina AV, George J, Senko O, *et al.* Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. *Cancer Res* 2006; **66**:10292-10301.
14 Ginestier C, Cervera N, Finetti P, *et al.* Prognosis and gene expression profiling of 20q13-amplified breast cancers. *Clin Cancer Res* 2006; **12**:4533-4544.
15 Malovannaya A, Lanz RB, Jung SY, *et al.* Analysis of the human endogenous coregulator complexome. *Cell* 2011; **145**:787-799.
16 O'Malley BW, Malovannaya A, Qin J. Minireview: nuclear receptor and coregulator proteomics--2012 and beyond. *Mol Endocrinol* 2012; **26**:1646-1650.
17 Sarai N, Nimura K, Tamura T, *et al.* WHSC1 links transcription elongation to HIRA-mediated histone H3.3 deposition. *EMBO J* 2013; **32**:2392-2406.
18 Wu SY, Chiang CM. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *J Biol Chem* 2007; **282**:13141-13145.
19 Dawson MA, Prinjha RK, Dittmann A, *et al.* Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* 2011; **478**:529-533.
20 Mochizuki K, Nishiyama A, Jang MK, *et al.* The bromodomain protein Brd4 stimulates G1 gene transcription and promotes progression to S phase. *J Biol Chem* 2008; **283**:9040-9048.
21 Delmore JE, Issa GC, Lemieux ME, *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011; **146**:904-917.
22 Herrmann H, Blatt K, Shi J, *et al.* Small-molecule inhibition of BRD4 as a new potent approach to eliminate leukemic stem- and progenitor cells in acute myeloid leukemia AML. *Oncotarget* 2012; **3**:1588-1599.
23 Zuber J, Shi J, Wang E, *et al.* RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 2011; **478**:524-528.
24 Groenendijk FH, Zwart W, Floore A, Akbari S, Bernards R. Estrogen receptor splice variants as a potential source of false-positive estrogen receptor status in breast cancer diagnostics. *Breast Cancer Res Treat* 2013; **140**:475-484.
25 Loven J, Hoke HA, Lin CY, *et al.* Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 2013; **153**:320-334.
26 Ott CJ, Kopp N, Bird L, *et al.* BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. *Blood* 2012; **120**:2843-2852.
27 Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, Brown M. Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res* 2007; **67**:6477-6483.
28 Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011; **474**:609-615.
29 Suarez L, Gore SD. Demethylation demystification. *Blood* 2013; **121**:1488-1489.
30 Daigle SR, Olhava EJ, Therkelsen CA, *et al.* Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell* 2011; **20**:53-65.
31 McCabe MT, Ott HM, Ganji G, *et al.* EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature* 2012; **492**:108-112.
32 Knutson SK, Wigle TJ, Warholic NM, *et al.* A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nat Chem Biol* 2012; **8**:890-896.
33 Lockwood WW, Zejnullahu K, Bradner JE, Varmus H. Sensitivity of human lung adenocarcinoma cell lines to targeted inhibition of BET epigenetic signaling proteins. *Proc Natl Acad Sci USA* 2012; **109**:19408-19413.
34 Mertz JA, Conery AR, Bryant BM, *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci USA* 2011; **108**:16669-16674.
35 Knowlden JM, Hutcheson IR, Jones HE, *et al.* Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF7 cells. *Endocrinology* 2003; **144**:1032-1044.
36 Pei H, Zhang L, Luo K, *et al.* MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature* 2011; **470**:124-128.
37 Feng Q, Yi P, Wong J, O'Malley BW. Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. *Mol Cell Biol* 2006; **26**:7846-7857.
38 Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012; **490**:61-70.
39 Kessler JD, Kahle KT, Sun T, *et al.* A SUMOylation-dependent transcriptional subprogram is required for Myc-driven tumorigenesis. *Science* 2012; **335**:348-353.
40 Gibbons DL, Lin W, Creighton CJ, *et al.* Expression signatures of metastatic capacity in a genetic mouse model of lung adenocarcinoma. *PLoS One* 2009; **4**:e5401.
41 Massarweh S, Osborne CK, Jiang S, *et al.* Mechanisms of tumor regression and resistance to estrogen deprivation and fulvestrant in a model of estrogen receptor-positive, HER-2/neu-positive breast cancer. *Cancer Res* 2006; **66**:8266-8273.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)